PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 5/10, 15/63, 15/86, C12P 21/00

A1

(11) International Publication Number: WO 95/16772

(43) International Publication Date: 22 June 1995 (22.06.95)

(21) International Application Number: PCT/US94/14502

(22) International Filing Date: 14 December 1994 (14.12.94)

(30) Priority Data:
08/166,925 14 December 1993 (14.12.93) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/166,925 (CIP)
Filed on 14 December 1993 (14.12.93)

(71) Applicant *(for all designated States except US)*: CORNELL RESEARCH FOUNDATION, INC. [US/US]; Suite 105, 20 Thornwood Drive, Ithaca, NY 14850 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: FALCK-PEDERSEN, Erik, S. [US/US]; 1161 York Avenue, New York, NY 10021 (US).

(74) Agents: GOLDMAN, Michael, L. et al.; Nixon, Hargrave, Devans & Doyle, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).

(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: ADENOVIRUS GENE EXPRESSION SYSTEM

(57) Abstract

The present invention provides a novel, highly efficient, recombinant adenovirus expression system for expression of a heterologous gene(s) and/or gene product(s) in a mammalian cell. The recombinant adenovirus was produced by cotransfecting a novel vector with the large fragment of the adenovirus-5 genome in 293 cells. Homologous recombination between these two DNA fragments resulted in the production of the recombinant adenovirus expression system. This vector, when converted to a recombinant virus has the unique capability of expressing one or more heterologous genes at very high levels. The novel vector, comprises, at least one cDNA insertion site for cloning a selected heterologous gene; a promoter sequence positioned upstream from the gene insertion site; the left end replication and packaging elements of the adenovirus-5 genome positioned upstream of the promoter; a highly efficient eukaryotic splice acceptor and splice donor site positioned immediately downstream of the promoter; and positioned downstream of the insertion site a strong polyadenylation sequence and the region for homologous recombination containing a portion of the adenovirus-5 genome. Between the packaging sequence and the CMV promoter are restriction sites for insertion of a second fully functional transcription unit.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

ADENOVIRUS GENE EXPRESSION SYSTEM

FIELD OF THE INVENTION

The present invention relates generally to a recombinant viral expression system. More particularly, the present invention relates to a highly efficient, recombinant adenovirus expression system capable of expressing a heterologous gene(s) in a host mammalian cell.

BACKGROUND OF THE INVENTION

The human adenovirus-5 (Ad5) genome consists of a double-stranded linear DNA molecule of 36 kilo-basepair (bp) (Ginsberg, 1984). The virus replication cycle has two phases: an early phase, during which 4 transcription units E1, E2, E3, and E4 are expressed, and a late phase occurring after the onset of viral DNA synthesis when late transcripts are expressed from the major late promoter (MLP). These late messages encode most of the viral structural proteins. E1, E2 and E4 gene products of human adenoviruses are involved in transcriptional activation, cell transformation, and viral DNA replication as well as other viral functions, and are essential for viral growth (Grand, 1987, *Biochem. J.*, vol. 241, pp. 25-38; and Nevins, 1987, *Microbiol. Rev.*, vol. 51, pp. 419-430). In contrast, E3 gene products are not required for viral replication in cultured cells (Ginsberg et al., 1989,), but appear to be involved in evading immune surveillance in vivo (Anderson et al., 1985, *Cell*, vol. 43, pp. 215-222; Burgert et al., 1985, *Cell*, vol. 41, pp. 987-997; Burgert et al., 1987, *EMBO J.*, vol. 6, pp. 2019-2026; Carlin et al., 1989, *Cell*, vol. 57, pp. 135-144; Gooding and Wold, 1990, *Crit. Rev. Immunol.*, vol. 10, pp. 53-71; Gooding et al., 1988, *Cell*, vol. 53, pp. 341-346; Horton et al., 1990, *J. Virol.*, vol. 64, pp. 1250-1255; Tollefson et al., 1991, *J. Virol.*, vol. 65, pp. 3095-3105; Wold and Gooding, 1989, *Mol. Biol. Med.*, vol. 6, pp. 433-452; and Wold and Gooding, 1991, *Virology*, vol. 184, pp. 1-8).

E1 and E3 and a site upstream of E4 have been utilized as sites for insertion of foreign DNA sequences in the generation of recombinant adenoviruses (Berkner et al.,

1984, *Nuc. Acids. Res.*, vol. 12, pp. 1925-1941; Chanda et al., 1990, *Virology*, vol. 175, pp. 535-547; Haj-Ahmad et al., 1986, *J. Virol.*, vol. 57, pp. 267-274; and Saito et al., 1985, *J. Virol.*, vol. 54, pp. 711-719). Since the upper size limit for DNA molecules that can be packaged into adenovirus particles is approximately 105% of the wild-type genome (Ghosh-Choudhury et al., 1987, *EMBO J.*, vol. 6, pp. 1733-1739), only about 2 kb of extra DNA can be inserted without compensating deletions of viral DNA. Although E1 is essential for virus replication in cell culture, foreign DNA can be substituted for E1 sequences when the virus is grown in 293 cells which are transformed by adenovirus-5 DNA and constitutively express E1 (Graham et al., 1977, *J. Gen. Virol.*, vol. 36, pp. 59-72). Several vectors having 1.9 kb deleted from E3 of adenovirus-5 have been constructed without interfering with virus replication in cell culture (Graham et al., 1992, Vaccines; New Approaches to Immunological Problems, R.W. Ellis (Ed.), Butterworth-Heinemann, Boston, MA, pp. 364-390). Such vectors allow for insertion of up to 4 kb of foreign DNA. Recombinant adenoviruses containing inserts in E3 replicate in all adenovirus-permissive cell lines and may be suitable as live recombinant viral vaccines since a number of adenovirus vectors containing E3 inserts have been shown to express foreign genes efficiently both *in vitro* and *in vivo* (Berkner, 1988: Chanda et al., 1990; Dewar et al., 1989, *J. Virol.*, vol. 63, pp. 129-136; Graham, 1990, *Trends Biotechnol.*, vol. 8, pp. 85-87; Graham et al., 1992; Johnson et al., 1988, *Virology*, vol. 164, pp. 1-14; Lubeck et al., 1989, *Proc. Natl. Acad. Sci. USA*, vol. 86, pp. 6763-6767; McDermott et al. 1989, *Virology*, vol. 169, pp. 244-247; Morin et al., 1987, *Proc. Natl. Acad. Sci. USA*, vol. 84, pp. 4626-4630; Prevec et al., 1989, *J. Gen. Virol.*, vol. 70, pp. 429-434; Prevec et al., 1990, *J. Inf. Dis.*, vol. 161, pp. 27-30; Schneider et al., 1989, *J. Gen. Virol.*, vol. 70, pp. 417-427; Vernon et al., 1991, *J. Gen. Virol.*, vol. 72, pp. 1243-1251; and Yuasa et al., 1991, *J. Gen. Virol.*, vol. 72, pp. 1927-1934).

Adenoviruses are good mammalian cell expression vectors with potential utility as live recombinant vaccines, in gene therapy, or for high level protein production in mammalian cells.

Adenovirus expression vectors have been in use for the past decade (Thummel et al., 1981, *Cell*, vol. 23, pp. 825-836; Berkner et al., 1984, *Nucleic Acids Res.*, vol. 12, pp. 1925-1941; and for a review see Grunhaus et al., 1992, *Seminars in Virology* 3, pp. 237-252), and more recently exploited for the purpose of gene therapy (Herz et al., 1993, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 90, pp. 2812-2816; Rosenfeld et al., 1991, *Science*, vol. 252, pp. 431-434; and Rosenfeld et al., 1992, *Cell*, vol. 68, pp. 143-155). Features of adenovirus based expression vectors which make them attractive to gene therapy applications include very efficient uptake into cells which contain the appropriate adenovirus receptor and uptake pathway, and the ability to carry up to 7.5 kb of foreign DNA. Adenovirus vectors allow a reporter gene to be under the control of tissue specific promoter elements (Friedman et al., 1986, *Mol. Cell. Biol.*, vol. 6, pp. 3791-3797; and Babiss et al., 1986, *Mol. Cell. Biol.*, vol. 6, pp. 3798-3806) as well as a variety of viral and mammalian constitutive promoter elements (Mittal et al., 1993, *Virus Research*, vol. 28, pp. 67-90).

One such example of an adenovirus-based vector system is described in Mittal et al., 1993, *Virus Research*, vol. 28, pp. 67-90. The authors here describe a helper-independent adenovirus type 5-luciferase recombinant containing the firefly luciferase gene flanked by simian virus 40 (SV40) regulatory sequences inserted into the early region 3 (E3) of the adenovirus-5 genome. A plasmid containing the luciferase gene and SV40 regulatory sequences in the E3 region was co-transfected with a plasmid containing the adenovirus-5 d1309 genome in circular form. Upon transfection of 293 cells, virus progeny produced by *in vivo* recombination between the two plasmids resulted in rescue of the adenovirus type 5-luciferase recombinant (i.e., E3 insert in Adenovirus-5 genome).

Gomez-Foix et al., 1992, *J. Biol. Chemistry*, vol. 267, no. 35, pp. 25129-25134, discloses adenovirus-mediated transfer of the muscle glycogen phosphorylase gene into hepatocytes in culture. The preparation of a recombinant adenovirus containing the cDNA encoding rabbit muscle glycogen phosphorylase is described whereby the cytomegalovirus (CMV) early gene promoter/enhancer, pUC 18 polylinker, fragment of the SV40 genome that includes the small T-antigen intron and the polyadenylation

signal, and cDNA that includes all of the protein coding region of the rabbit muscle glycogen phosphorylase, was inserted into vector pAC. The resulting plasmid was co-transfected into 293 cells with plasmid pJM17, which encodes a full-length adenovirus-5 genome. Homologous recombination between the recombinant plasmids in 293 cells generated a genome of packageable size in which the adenovirus early region 1 was replaced by the cloned chimeric gene encoding rabbit muscle glycogen phosphorylase.

Roessler et al., 1993, *J. Clin. Invest.*, discloses using a recombinant adenoviral vector for the expression of the gene for *Escherichia coli* beta-galactosidase within synovium tissue. Replication defective adenoviral vectors are deleted of sequences spanning E1A, E1B and a portion of the E3 region, impairing the ability of this virus to replicate or transform nonpermissive cells. The early enhancer/promoter of the cytomegalovirus (CMV) was inserted into this vector to drive transcription of *lacZ* with a SV40 polyadenylation sequence cloned downstream from this reporter.

Yang et al., *Proc. Natl. Acad. Sci. USA*, vol. 90, pp. 9480-9484, discloses the expression of cystic fibrosis transmembrane conductance regulator (CFTR) by adenovirus-mediated gene transfer. The recombinant adenoviruses were produced by homologous recombination of two vectors which contain the following relevant sequences: 5' ITR of adenovirus-5 spanning 0-1 map units; *Tha* I-*Sna*BI fragment of the immediate-early gene of cytomegalovirus; promoter from the chicken β-actin gene spanning *Xho* I at nucleotide (nt) -275 to *Mbo* I at nt +1; human CFTR cDNA containing 60 nt of 5' untranslated sequence, the entire coding sequence, and 80 nt of 3' untranslated sequence; simian virus 40 late gene polyadenylation signal; 9.2-16.1 map units of adenovirus-5; and plasmid sequences.

Herz et al., 1993, *Proc. Natl. Acad. Sci. USA*, vol. 90, pp. 2812-2816, discloses the use of adenovirus-mediated gene transfer to transiently elicit production of low density lipoprotein (LDL) receptors in mice. Recombinant adenoviruses containing: 1) cDNA encoding the human LDL receptor (AdCMV-LDLR)(CMV,cytomegalovirus); 2) β-galactosidase (AdCMV-βgal); and firefly luciferase (AdCMV-Luc), were prepared using co-transfection of the appropriate plasmids in 293 cells.

Rosenfeld et al., 1991, *Science*, vol. 252, pp. 431-434, discloses adenovirus-mediated transfer of recombinant α1-antitrypsin gene to the lung epithelium cells of the cotton rat respiratory tract *in vivo*. The adenoviral vector contained an adenovirus major late promoter and a recombinant human α1-antitrypsin gene.

Quantin et al., 1992, *Proc. Natl. Acad. Sci. USA*, vol. 89, pp. 2581-2584, discloses a recombinant adenovirus containing the β-galactosidase reporter gene under the control of muscle-specific regulatory sequences. This recombinant virus directed expression of the β-galactosidase in myotubes *in vivo*.

Problems associated with adenovirus infection, particularly those associated with repression of host cell mRNA translation and shutdown of host normal mRNA production (Babich et al., 1983, *Mol. Cell. Biol.*, vol. 3, pp. 1212-1221; Beltz et al., 1979, *J. Mol. Biol.*, vol. 131, pp. 353-373; Schneider et al., 1987, *Annu. Rev. Biochem.*, vol. 56, pp. 317-332) have been addressed by using defective adenovirus vectors which are based on mutations in the dominant regulatory region, E1 (Harrison et al., 1977, *Virology*, vol. 77, pp. 319-329; Jones et al., 1979, *Cell*, vol. 17, pp. 583-689). In addition, conventional adenovirus vector systems typically require high cell exposure (e.g., MOI's in excess of 500 PFU/cell) for expression of the desired gene, which is detrimental to the cells because of cytopathic effects from exposure. Therefore, a need exists for an adenovirus-mediated expression vector which can infect cells at low doses, yet can exhibit maximum expression of a gene in the cell.

Moreover, although adenovirus-based vectors for gene expression have been successfully employed with a number of mammalian and viral genes (for review, see Mulligan, R.C., 1993, *Science*, vol. 260, pp. 926-932), they have not apparently been used to express any member of the guanine nucleotide-binding protein coupled receptors (GPCR) family, such as the pituitary thyrotropin-releasing hormone (TRH-R)(Straub et al., 1990, *Proc. Natl. Acad. Sci U.S.A.*, vol. 87, pp. 9514-9518; Yamada et al., 1992, *Biochem. Biophys. Res. Commun.*, vol. 184, pp. 367-372; Zhao et al., 1992, *Endocrinology*, vol. 130, pp. 3529-3536; de la Pena et al., 1992, *Biochem. J.*, vol. 284, pp. 891-899). Seven transmembrane-spanning GPCRs comprise a large family of cell surface regulatory proteins (Dohlman et al., 1991, *Annu. Rev. Biochem.*,

vol. 60, pp. 653-688). When studying the molecular details of receptor biology in mammalian cells, expression of wild type and mutant receptors is usually accomplished by gene transfer by one of several transfection procedures.

Assays using 1) a cell system that permits intracellular replication of the plasmid vector during transient expression studies; or 2) that stably express the receptor of interest, provide useful, but, limited receptor expression. Where transfections yield low levels of receptor expression, or where the range of cell types that can be transfected is restricted, studies of these receptors is limited. Adenovirus-mediated gene transfer could be employed as an alternative strategy to plasmid based receptor expression vectors. A significant advantage of using adenovirus-mediated gene transfer is the wide variety of cells which are susceptible to infection by adenovirus. This should permit study of TRH-R biology in a variety of mammalian cell types, including those not amenable to transfection techniques.

Furthermore, the analysis of elements involved in cardiac myocyte gene regulation would be greatly facilitated by a simple and efficient method of adenovirus-mediated gene transfer. Because there are no permanent cardiac myocyte cell lines, the majority of cardiac myocyte gene expression studies have been carried out using transient gene transfer techniques into primary cultures of fetal and neonatal cardiocytes (Gustafson et al., 1987, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 84, pp. 3122-3126). Although useful, this methodology has many limitations, including relatively low efficiency as well as being restricted to fetal and neonatal stages of development since transient transfection of adult cardiac myocytes has not been reported.

As an alternative, *in vitro* studies of cardiac myocyte gene regulation and gene transfer have been successfully carried out in transgenic (Rindt et al., 1993, *J. Biol. Chem.*, vol. 268, pp. 5332-5338; and Subramanian et al., 1991, *J. Biol. Chem.*, vol. 266, pp. 24613-24620). However, the generation of transgenic mouse lines is both costly and extremely time consuming.

A second approach to cardiac gene transfer *in vitro* has relied on injecting plasmid DNA into the myocardium and measuring reporter gene expression in the cells

which have successfully taken up sufficient quantities of DNA (Kitsis et al., 1991, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 88, pp. 4138-4142; Lin et al., 1990, *Circulation*, vol. 82, pp. 2217-2221; and Ascadi et al., 1991, *The New Biologist*, vol. 3, pp. 71-81). The problem associated with direct DNA injection is its relative inefficiency as only approximately 0.02% of the myocytes in the adult rat heart take up and express injected DNA (Kitsis et al., 1993, in *Methods in Molecular Genetics*, ed. Adolph, K. W., Academic Press, Inc., New York, Vol. 1, pp. 374-392).

A recent report demonstrated efficient gene transfer into adult rat cardiocytes *in vitro* (Kirshenbaum et al., 1993, *J. Clin. Invest.*, vol. 92, pp. 381-387). In addition, recent studies using adenovirus vectors introduced intravenously into both rats and mice, indicate that the virus will infect a wide variety of tissue types, including mouse skeletal and cardiac muscle (Quantin et al., 1992, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 89, pp. 2581-2584; and Strattford-Perricaudet et al., 1992, *J. Clin. Invest.*, vol. 90, pp. 626-630). However, little quantitative data is available concerning expression of adenovirus-mediated gene transfer *in vivo*. Therefor, a need exists for an adenovirus-mediated gene transfer vector system which would function effectively with primary cultures of cardiac myocytes and one which would also have application *in vitro*.

SUMMARY OF THE INVENTION

The primary object of the present invention is to provide an adenovirus-based expression system capable of expressing a heterologous gene(s) in a host mammalian cell.

The present invention provides a novel, highly efficient, recombinant adenovirus expression system for expression of a heterologous gene(s) and/or gene product(s) in a mammalian cell. The recombinant adenovirus expression system of the invention was produced via homologous recombination between the novel vector of the invention co-transfected with the large fragment of the adenovirus-5 genome in 293 cells.

In accordance with the present invention, the novel expression vector is preferably a plasmid vector. The plasmid vector of the invention can be used as a generic vector, that is, for the expression of any number of selected heterologous gene(s). The generic plasmid vector is designated pAdCMV-HS-Vector. The plasmid vector described herein can itself be transfected into a mammalian cell for the expression of any number of gene(s) and/or production of a gene product(s), depending on the heterologous gene(s) cloned into the plasmid vector. Alternatively, the plasmid vector can be converted into the recombinant adenovirus of the invention. Examples of various uses of the plasmid vector are described in the various embodiments disclosed herein.

In one embodiment of the invention, the plasmid vector includes at least one cDNA insertion site, i.e., restriction site(s) for cloning a selected heterologous gene(s). Positioned upstream of the gene insertion site(s) is a promoter which controls expression of the heterologous gene(s). The promoter is preferably the mouse cytomegalovirus (CMV) early promoter, or an effective expression promoting fragment thereof. Positioned upstream of the promoter, is the left end replication and packaging elements of the adenovirus-5 genome. A eukaryotic splice acceptor and splice donor site is positioned immediately downstream of the promoter.

Following the splicing sequence elements, is the gene insertion site(s), which is followed by the polyadenylation sequence, and the region for homologous recombination which contains a portion of the adenovirus-5 genome. The polyadenylation sequence preferably comprises the 3' processing site taken from the mouse β–globin transcription unit i.e., Globin poly(A). The order and choice of the splicing and polyadenylation elements results in optimal processing of the pre-mRNA into mRNA. The region for homologous recombination preferably is the adenovirus-5 genome nucleotide sequence 2800-5776.

The plasmid vector of the invention can be readily converted into a recombinant adenovirus for expression of a heterologous gene(s) and/or gene product(s) in a mammalian cell. Here, the plasmid vector is co-transfected with the large fragment of the adenovirus-5 genome i.e., 3.8-100 map units and/or an appropriate

derivative thereof. Homologous recombination between these DNA fragments results in the production of a replication defective, recombinant adenovirus. The recombination reconstructs the adenovirus-5 genome by displacing the E1A and E1B protein coding regions with the plasmid vector cDNA.

In another embodiment of the invention, there is provided a recombinant adenovirus expression system for the receptor for thyrotropin-releasing hormone (TRH-R). The recombinant adenovirus, designated AdCMVmTRHR, circumvents difficulties encountered when using conventional transient or stable plasmid expression systems. Using this recombinant adenovirus (AdCMVmTRHR), TRH-Rs can be expressed in different mammalian cell types, including those resistant to transient transfection assay. Recombinant adenovirus, AdCMVmTRHR, was produced by homologous recombination between plasmid vector, designated pAdCMVmTRHR, i.e. the generic plasmid vector of the invention containing the gene coding TRH-R, co-transfected with the large fragment of adenovirus-5 d1309 genome. The versatility of using adenovirus mediated gene transfer and expression of TRH-Rs not only facilitates *in vitro* studies of TRH-R biology, but provides a valuable *in vivo* expression vector capable of extending TRH-R studies in animal model systems.

In a further embodiment of the present invention, infection of cultured fetal and adult rat cardiac myocytes *in vitro* and of adult cardiac myocytes *in vivo* was characterized using the recombinant adenovirus of the invention. The recombinant adenovirus, designated AdCMVCATgD, includes the chloramphenicol acetyltransferase (CAT) reporter gene driven by the cytomegalovirus (CMV) promoter. Plasmid vector pAdCMVCATgD i.e., generic plasmid vector of the present invention containing the gene encoding the bacterial CAT sequence, was co-transfected with the large fragment of the adenovirus-5 genome (3.6-100 map units). Homologous recombination between the plasmid vector and adenovirus fragment produced the recombinant adenovirus, designated AdCMVCATgD.

Virtually all fetal or adult cardiocytes expressed the CAT gene *in vitro* when infected with 1 plaque forming unit (pfu) of virus per cell. Using *in vitro* studies as a guide, recombinant virus AdCMVCATgD was introduced directly into adult rat

myocardium and the expression results obtained from virus injection was compared to those obtained by direct injection of plasmid vector pAdCMVCATgD DNA. The amount of CAT activity resulting from adenovirus infection of the myocardium is orders of magnitude higher than that seen from DNA injection and is proportional to the amount of input virus. The recombinant adenovirus-mediated gene delivery system is a very effective tool for high efficiency gene transfer into the cardiovascular system.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1(a) & 1(b) is a graphic map showing the structure of the generic plasmid vector of the invention, designated pAdCMV-HS-Vector.

Fig. 2 is a graphic map showing the structure of plasmid vector pGEM2AdCMV.

Fig. 3 is a graphic map showing the structure of plasmid vector ML SIS CAT.

Fig. 4 is a graphic map showing the structure of plasmid vector ML SIS CAT-PA #11.

Fig. 5 is a graphic map showing the structure of plasmid vector ML SIS CAT gD355.

Fig. 6 is a graphic map showing the structure of plasmid vector pAdCMVCATgD.

Fig. 7 is a graphic map showing the structure of plasmid vector pPYNeo.

Fig. 8 is a graphic map showing the structure of plasmid vector pMLAdCMVCATgDNeo-.

Fig. 9 is a graphic map showing the structure of plasmid vector pAdCMVdH-TRHRE2.

Fig. 10 is a graphic map showing the structure of plasmid vector pAdCMVdH-IFN-GL3.

Fig. 11 is a graphic map showing the structure of plasmid vector pGEM2AdCMVcatgD.

Fig. 12 is a graphic map showing the structure of plasmid vector pML-E1aEF-5778.

Fig. 13 is a graphic map of plasmid vector pAdCMVmTRHR used for the construction of recombinant adenovirus AdCMVmTRHR.

Fig. 14 is a graph showing a comparison of infection with AdCMVmTRHR and transfection with pAdCMVmTRHR on expression of TRH-Rs and *methyl*TRH responsiveness in six mammalian cell lines.

Fig. 15 is a graph showing TRH-induced desensitization and PMA-induced inhibition of the TRH response in AdCMVmTRHR-infected GHY, COS-1 and KB cells.

Fig. 16 is a graph showing *methyl*TRH-stimulated TRH-R internalization in AdCMVmTRHR-infected GHY, COS-1 and KB cells.

Fig. 17 is a graphic map of plasmid vector pAdCMVCATgD used in construction of recombinant adenovirus AdCMVCATgD.

Fig. 18(a) is a graph showing dosage and time dependent expression of adenovirus in fetal cardiocytes.

Fig. 18(b) is a graph showing dosage and time dependent CAT expression following infection by AdCMVCATgD in adult cardiocytes.

Fig. 19 is a graph showing distribution of CAT activity in cells of AdCMVCATgD injected hearts.

Fig. 20A is a graph showing CAT expression in the left ventricle 5 days following intracardiac injection of four doses of adenovirus [AdCMVCATgD; $6x10^6$, (n=4); $6x10^7$, (n=4); $6x10^8$, (n=3); and $2x10^9$, (n=2)].

Fig. 20B is a graph showing CAT expression over time in the left ventricle following injection of $6x10^7$ pfu of AdCMVCATgD virus.

Fig. 21 (a-f) is an immunohistochemical staining for CAT protein in adenovirus infected hearts.

Fig. 22 is a schematic showing the nucleotide sequence of plasmid vector pAdCMV-HS-Vector, as shown in Figs. 1(a) & 1(b).

Fig. 23 is a schematic showing the nucleotide sequence of another version of plasmid vector pAdCMV-HS-vector.

DETAILED DESCRIPTION OF THE INVENTION

As used throughout this specification, the following definitions apply for purposes of the present invention:

The term "restriction enzyme digestion" of DNA refers to catalytic cleavage of the DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction endonuclease, and the sites for which each is specific is called a restriction site. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements as established by the enzyme suppliers are used. Restriction enzymes commonly are designated by abbreviations composed of a capital letter followed by other letters representing the microorganism from which each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1 μg of plasmid or DNA fragment is used without 1-2 units of enzyme in about 20 μl of buffer solution. Appropriate buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation of about 1 hour at 37^0C is ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein or polypeptide is removed by extraction with phenol and chloroform, and the digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme may be followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction cleaved ends of a DNA fragment from circularizing or forming a closed loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional as described in Sections 1.56-1.61 of Sanbrook, et.al., *Molecular Cloning: A Laboratory Manual* New York: Cold Spring Harbor Laboratory Press, 1989, which disclosure is hereby incorporated by reference).

The term "recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by

electrophoresis, identification of the fragment of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. These procedures are generally well known. For example, see Lawn et al., 1981, *Nucleic Acids Res.*, vol. 9, pp. 6103-6114; and Goeddel et al., 1980, *Nucleic Acids Res.*, vol. 8, p. 4057, which disclosures are hereby incorporated by reference.

The term "expression" may be characterized as follows: A cell is capable of synthesizing many proteins. At any given time, many proteins which the cell is capable of synthesizing are not being synthesized. When a particular polypeptide, coded for by a given gene, is being synthesized by the cell, that gene is said to be expressed. In order to be expressed, the DNA sequence coding for that particular polypeptide must be properly located with respect to the control region of the gene. The function of the control region is to permit the expression of the gene under its control.

The term "southern blot analysis" is a method by which the presence of DNA sequences in a restriction endonuclease digest of DNA or DNA-containing composition is confirmed by hybridization to a known, labeled oligonucleotide or DNA fragment. Southern analysis typically comprises electrophoretic separation of DNA digests on agarose gels, denaturation of the DNA after electrophoretic separation, and transfer of the DNA to nitrocellulose, nylon, or another suitable membrane supports for analysis with a radiolabeled, biotinylated or enzyme-labeled probe as described in sections 9.37-9.52 of Sambrook et al., supra, which disclosure is hereby incorporated by reference.

The term "northern analysis" is a method used to identify RNA sequences that hybridize to a known probe such as an oligonucleotide, DNA fragment, cDNA or fragment thereof, or RNA fragment. The probe is labeled with a radioisotope such as ^{32}P, or by biotinylation, or with an enzyme. The RNA to be analyzed is usually electrophoretically separated on an agarose or poly-acrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe, using standard techniques well known in the art, such as those described in sections

7.39-7.52 of Sambrook et al., supra, which disclosure is hereby incorporated by reference.

The term "vector" refers to an extra-chromosomal molecule of duplex DNA comprising an intact replicon that can be replicated in a cell. Generally, vectors are derived from viruses or plasmids of bacteria and yeasts. An adenovirus vector comprises an adenovirus replicon.

The term "gene" refers to those DNA sequences which transmit the information for and direct the synthesis of a single protein chain.

The term "infection" refers to the invasion by agents (e.g., viruses, bacteria, etc.) of cells where conditions are favorable for their replication and growth.

The term "heterologous gene" in reference to the adenovirus vectors hereof, refers to DNA that encodes polypeptides ordinarily not produced by the virus from which the vector is derived, but which is introduced into the cell as recombinant DNA or within viruses carrying recombinant DNA genomes.

The term "plasmid" means a bacterial vector which is used as an intermediate in the construction of a virus vector. A plasmid facilitates the transfer of exogenous genetic information, such as the combination of a novel promoter and a heterologous structural gene under the regulatory control of that promoter, to a specific site within the viral genome by homologous recombination via the DNA sequences flanking the chimeric gene. The plasmid can itself express a heterologous gene inserted therein.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed form such available plasmids in accord with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to one of ordinary skill in the art.

The term "ligation" means the process of forming phosphodiester bonds between two nucleic acid fragments. To ligate the DNA fragments together, the ends of the DNA fragments must be compatible with each other. In some cases, the ends will be directly compatible after endonuclease digestion to blunt ends to make them

compatible for ligation. To blunt the ends, the DNA is treated in a suitable buffer for at least 15 minutes at 15° C, with about 10 units of the Klenow fragment of DNA polymerase I or T4 DNA polymerase in the presence of the four deoxyribonucleotide triphosphates. The DNA is then purified by phenolchloroform extraction and ethanol precipitation. The DNA fragments that are to be ligated together are put in solution in about equimolar amounts. The solution will also contain ATP, ligase buffer, and a ligase such as T4 DNA ligase at about 10 units per 0.5 g of DNA. If the DNA is to be ligated into a vector, the vector is first linearized by digestion with the appropriate restriction endonuclease(s). The linearized fragment is then treated with bacterial alkaline phosphatase, or calf intestinal phosphatase to prevent self-ligation during the ligation step.

"Preparation" of DNA from cells means isolating the plasmid DNA from a culture of the host cells. Commonly used methods for DNA preparation are the large and small scale plasmid preparations described in sections 1.25-1.33 of Sambrook et al., supra, which disclosure is hereby incorporated by reference. After preparation of the DNA, it can be purified by methods well known in the art such as that described in section 1.40 of Sambrook et al., supra, which disclosure is hereby incorporated by reference.

This invention achieves significantly enhanced *in vitro* and *in vivo* expression levels of heterologous gene(s) by inserting into a host mammalian cell the adenovirus expression system or plasmid vector of the invention, containing foreign cDNA encoding the heterologous gene(s) under the transcriptional control of DNA fragments derived from the mouse cytomegalovirus (CMV) immediate early gene regulatory sequences. It is understood that the CMV immediate early promoter can be combined with enhancer elements isolated from other transcriptional units to increase expression efficiency.

The recombinant adenovirus expression system and plasmid vector include at least one cDNA insertion site(s) i.e., restriction site(s) for cloning a selected heterologous gene(s). Other important features of the adenovirus expression system and plasmid vector of the invention include a highly efficient eukaryotic splicing

sequence elements located immediately downstream the promoter, and a strong polyadenylation sequence following the heterologous gene insertion site.

In an alternative embodiment of the invention, the plasmid vector can be readily converted into the recombinant adenovirus expression system of the invention for expression of a heterologous gene(s) and/or gene product(s) in a mammalian cell. To produce the recombinant adenovirus, the plasmid vector of the invention is co-transfected with the large fragment of adenovirus-5 genome in 293 cells. Homologous recombination between these DNA fragments results in the production of a replication defective, recombinant adenovirus, which includes cDNA from the plasmid vector.

Host cells useful for expression of the heterologous gene(s) includes any mammalian cell in which the recombinant adenovirus and/or plasmid vector of the invention are capable of uptake and expression. The plasmid vector of the invention can be used to transfect a mammalian host cell for production of the inserted gene product. It is understood that the plasmid vector can be introduced into the host cell(s) using conventional techniques known in the art, such as, for example, transfection. The recombinant adenovirus can be introduced into the host cell via infection using standard techniques in the art.

The plasmid vector(s) and recombinant adenovirus(es) of this invention can be prepared using standard genetic engineering technologies known to the art, as described by Maniatis et al., 1982, *Molecular Cloning: A Laboratory Manual*, Cold Springs Laboratory, Cold Springs Harbor, New York; and Sambrook et al., (*Molecular Cloning: A Laboratory Manual* New York: Cold Spring Harbor Laboratory Press, 1989, which disclosures are hereby incorporated by reference.

In a preferred embodiment of the invention, the plasmid vector of the invention comprises, starting from the left end of the adenovirus at position 1, adenovirus nucleotide sequence from 1-353 containing the origin of replication and the viral packaging sequence; adenovirus nucleotide sequence from 354-2800 was deleted and replaced with the CMV-1 promoter, eukaryotic splice elements, the cDNA encoding the selected heterologous gene(s) and the Globin poly(A) site; and adenovirus

nucleotide sequence from nucleotide 2800-5776, which serves as the region for homologous recombination.

To obtain efficient expression of the heterologous gene(s), a eukaryotic promoter must be present in the plasmid vector and recombinant adenovirus expression system. It is understood that any known eukaryotic promoter can be utilized in the plasmid vector and/or recombinant adenovirus expression system of the invention provided the promoter is capable of expressing the heterologous gene(s). The promoter used herein, preferably, is the mouse cytomegalovirus-1 early promoter, or an effective expression promoting fragment thereof. For an example of the CMV promoter, see U.S. Patent No. 4,963,481 to Jean P. deVilliers, which disclosure is hereby incorporated by reference. The use of the mouse CMV promoter is of broad utility because this promoter has a very broad host range and functions with superior strength and efficiency in a wide variety of cell lines tested.

The presence and position of the splicing elements with respect to the cDNA are important to overall processing efficiency, as is the choice of splicing elements. In the present invention, a hybrid splice donor and acceptor was used which yielded a highly efficient processing activity compared to the more common splice element used in other systems i.e., the SV40 small T splice site. By inserting the cDNA downstream of the splice elements, we are coupling the splice elements to the downstream 3' processing site generating a terminal exon. Use of a demonstrably efficient poly(A) site maximizes efficiency of the expression system. This allows efficient conversion of pre-mRNA into mRNA and allows the system to take full advantage of the high level of expression generated by the CMV promoter.

Any of the conventional cloning methods for insertion of the gene and/or gene fragment(s) into the plasmid vector can be used to ligate the promoter and the other control elements into specific sites within the plasmid vector. Accordingly, heterologous gene sequence(s) containing those regions coding for the gene(s) can be ligated into the plasmid vector at a specific restriction site in relation to the promotor and control elements so that when either the recombinant adenovirus or plasmid vector

is introduced into the mammalian cell, the foreign genetic sequence can be expressed (i.e., transcribed and translated) by the host cell.

Another important feature of the adenovirus expression system and plasmid vector of the invention, is the ability to express more than one heterologous gene, simultaneously. Using the expression systems of the invention, it is possible to express at least two heterologous genes at the same time. The second heterologous gene, is preferably inserted into the *Not*1 restriction site in the plasmid vector. However, it is understood that other restriction sites positioned between the packaging sequence and the promoter are available for insertion of the second gene.

As previously mentioned, the plasmid vector can be introduced into an appropriate host cell (i.e., mammalian cells) by transfection, and the recombinant adenovirus can be introduced by infection. Stable transformants can be selected based upon the expression of one or more appropriate gene markers either present or inserted into the adenovirus plasmid, such as, for example, G418 resistance in eukaryotic host systems. Expression of such marker genes should indicate that the recombinant DNA molecule is integrated and functional. It is understood that any known gene marker in the art can be utilized herein. Such gene markers can be derived from cloning vectors, which usually contain a marker function.

The plasmid vector and recombinant adenovirus containing the heterologous gene(s) can be identified by three approaches: (1) DNA-DNA hybridization using probes comprising sequences that are homologous to the gene(s); (2) presence or absence of "marker" gene function and (3) expression of inserted sequences based on physical, immunological or functional properties. Once a recombinant which expresses the gene is identified, the gene product should be analyzed. One goal of the invention is to use the plasmid vector and recombinant adenovirus expression system for gene expression and/or gene transfer in mammalian cells. Once the recombinant virus or plasmid is identified, it is cultured under conditions which facilitate growth of the cells and expression of the gene as will be apparent to one skilled in the art. Thereafter, the gene product can be isolated and purified by standard methods including

chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard techniques.

The protein(s) encoded by the heterologous gene(s) inserted into the plasmid vector and recombinant adenovirus expression system can comprise any known protein, including; growth hormone, human growth hormone (HGH), des-N-methionyl human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin A-chain, insulin B-chain, proinsulin, relaxin A-chain, relaxin B-chain, prorelaxin, follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), leutinizing hormone (LH), glycoprotein hormone receptors, calcitonin, glucagon, factor VIII, an antibody, lung surfactant, urokinase, streptokinase, human tissue-type plasminogen activator (t-PA), bombesin, factor IX, thrombin, hemopoietic growth factor, tumor necrosis factor-alpha and -beta, enkephalinase, human serum albumin, mullerian-inhibiting substance, mouse gonadotropin-associated peptide, β-lactamase, tissue factor protein, inhibin, activin, vascular endothelial growth factor, integrin receptors, thrombopoietin, protein A or D, rheumatoid factors, NGF-β platelet-growth factor, transforming growth factor; TGF-alpha and TGF-beta insulin-like growth factor-I and -II, insulin-like growth factor binding proteins, CD-4, DNase, latency associated peptide erythropoietin, osteoinductive factors, interferon, alpha, - beta, and -gamma, colony stimulating factors (CSFs), M-CSF, GM-CSF, and G-CSF, interleukins (ILs), IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, superoxide dismutase; viral antigens; HIV envelope proteins GP120 and GP140, immuno globulins, and fragments of the above listed proteins.

The following Examples are provided to further illustrate the present invention.

Example I

Construction of pGEM2AdCMV

Plasmid pBstSK+ (0-353) contains adenovirus-5 sequence from nt 0-353 inserted into pBstSK+ vector at the *Eco*RI site and the *Sst*II site (which has been lost by blunt end ligation). These sequences are required viral elements which include the origin for DNA replication and the viral packaging sequence. The CMV

enhancer/promoter was taken from the plasmid CDM8 (INVITROGEN) by digestion with *Hind*III and *Hinc*II. *Not*I linkers were added to the *Hinc*II site followed by digestion with *Not*I and *Sst*I. Isolation of the resulting 592 bp fragment (CDM8 numbers 1533-2192) and insertion into pBstSK+ 0-353 vector at the *Not*I and *Sst*I sites gave the plasmid pBstSK+ 0-353-CMV. The *Eco*RI-*Sst*I fragment was isolated and inserted into the *Eco*RI-*Sst*I sites of pGEM 2 vector to generate pGEM2AdCMV, as shown in Figure 2. This construct has a polylinker from *Sst*I to *Hind*III available for cloning.

Example II

Construction of pMLSISCATgD

The SV40 poly A site was deleted from vector pMLSISCAT (Figure 3)(Huang et al., 1990, NAR, vol. 18, pp. 937-947, which disclosure is hereby incorporated by reference) by *Nar*I-*Kpn*I digestion and blunt end circularization of the plasmid to make pMLSISCAT(-pA). The mouse β-major globin poly A site was isolated by *Nar*I-*Sal*I digestion of pMLgDØ. This fragment was blunt end inserted into the *Bam*HI site of pMLSISCAT(-pA) (Figure 4) to create pMLSISCATgD (Figure 5).

Example III

Construction of pAdCMVCatD

pGEM2AdCMV (Figure 2) was digested with *Xba*I; pMLSISCATgD (Figure 5) was digested with *Xba*I and the fragment containing the splicing elements, the coding sequence for CAT and the globin poly(A) site was isolated and inserted into the *Xba*I site of pGEM2AdCMV to create pGEM2AdCMVCatgD (Figure 11). pGEM2AdCMVCatgD was digested with *Pvu*I and *Sal*I and the coding plasmid was isolated and inserted into vector pMLP6gEF also cut with *Pvu*I and *Sal*I. The plasmid was constructed into an intact replication defective adenovirus by co-transfecting the plasmid vector with the 3.6-100 m.u. large fragment of adenovirus in 293 cells.

Example IV
Construction of pAdCMVdHCatgD

One of the three *Hind*III restriction sites in pAdCMVCatgD (Figure 6) was deleted by partial *Hind*III digestion and filling by Klenow large fragment of DNA polymerase followed by plasmid circularization and ligation. This allowed removal of the CAT sequence and the poly(A) site by *Hind*III digestion, with the retention of promoter and splicing sequences. A 1100 bp of E1B sequence was deleted.

Example V
Construction of pAdCMVCatgDNeo(-)

The unique restriction site *Not*I located at position 361 can be used to insert any additional gene of interest. As a test construct a *Not*I fragment from pPYNeo was isolated which contained the Neomycin resistance gene driven by the polyoma promoter and using the SV40 splicing and polyadenylation elements. This strategy resulted in the introduction of the Neo gene into the vector in two orientations relative to the direction of CAT gene expression (+) and (-). Both of these constructs were used in virus constructions, however, only the AdCMVCatDNeo(-) virus has been isolated to date.

Example VI
Construction of pAdCMVTRHrE3

Using vector pAdCMVdHCatgD (Figure 6), cDNA for thyrotropin releasing hormone receptor (which contains the adenovirus E2 poly(A) site) was inserted directly into the *Hind*III digested vector to construct vector pAdCMVTRHrE3 (Figure 9).

Example VII

Construction of pAdCMV-Gamma Interferon L3

The cDNA for gamma interferon (with the added poly(A) site from adenovirus major late L3) was inserted into the pAdCMVdHCatgD (Figure 6) *Hind*III digested vector to construct vector pAdCMVdH-IFN-GL3.

Example VIII

Construction of pAdCMV-HS-Vector

pAdCMV-HS-Vector (Figures 1(a) & 1(b)) has the globin poly(A) site inserted downstream of the L3 poly(A) site of pAdCMV-gamma interferon. Digestion with *Hind*III and *Sal*I released the Interferon cDNA and the L3 poly(A) site leaving the Adenovirus 0-353 sequence, the CMV promoter, the splice acceptor and donor, and the globin poly(A) site and adenovirus sequence from 2800-5776. With reference to Figures 22 and 23, there is shown the nucleotide sequences of two versions of plasmid vector pAdCMV-HS-Vector.

Example IX

Expression of Thyrotropin-Releasing Hormone (TRH) Receptors

1.Materials:

Dulbecco's modified Eagle's medium, modified Eagle's medium, Ham's F10 medium and horse and fetal bovine serums were purchased from GIBCO. Nu-serum was obtained from Collaborative Research. TRH, *methyl*TRH and PMA were obtained from SIGMA. *myo*-[^{3}H]inositol was obtained from Amersham. [^{3}H]*methyl*TRH was obtained from Du Pont-New England Nuclear. The expression vector pCDM8 was obtained from INVITROGEN.

2. Construction of AdCMVmTRHR:

The parent plasmid, pAdCMVmTRHR, was constructed by inserting a 1.2 kb *Eco*RI-*Not*I fragment containing the protein-coding region of the mouse TRH-R cDNA, nucleotides 233-1462 of plasmid pBSmTRHR (Straub et al., 1990, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 87, pp. 9514-9518, which disclosure is hereby incorporated by reference), into plasmid pGEM2-L3-114 at the *Eco*RI-*Bam*HI site. After digesting with *Eco*RI and using the Klenow fragment of DNA polymerase I to make blunt DNA ends, *Hind*III linkers were ligated and a 1.4 kb *Hind*III fragment containing mouse TRH-R cDNA and the adenovirus E2 poly(A) signal sequence was isolated and inserted into the *Hind*III site of the pAdCMV-HS-Vector (i.e., expression plasmid of the present invention) which contains the left end replication and packaging elements of adenovirus, the cytomegalovirus-1 promoter and splicing elements from plasmid pML-IS Cat (Huang et al., 1990, *Nucleic Acids Res.*, vol. 18, pp. 937-947, which disclosure is hereby incorporated by reference). Following verification of the plasmid by restriction site mapping and transient transfection of pAdCMVmTRHR into COS-1 cells to demonstrate TRH-R expression, the virus AdCMVmTRHR was constructed by overlap recombination as described by Tantravahi et al., 1993, *Mol. Cell. Biol.*, vol. 13, pp. 578-587, which disclosure is hereby incorporated by reference. All transfections were carried out in human embryonic kidney cells transformed with the E1 region of adenovirus type 5 according to the procedure of Graham et al., 1977, *J. Gen. Virol.*, vol. 36, pp. 59-72, which disclosure is hereby incorporated by reference. Following plaque purification, virus was grown in 293 cells in suspension cultures as described by Antravahi et al., 1993, *Mol. Cell. Biol.*, vol. 13, pp. 578-587, which disclosure is hereby incorporated by reference. The entire sequence coding for the adenovirus E1a gene was removed as well as the 5' 1.8 kb of the E1b gene. Co-transfection of pAdCMVmTRHR with the large fragment of adenovirus (3.8-100 map units) into 293 cells resulted in production of recombinant virus AdCMVmTRHR.

3. Infection with AdCMVmTRHR:

Cells were seeded in wells (3.8 cm^2) pretreated with poly-L-lysine and were incubated in medium supplemented with serum in a humidified atmosphere of 5% CO_2. After a minimum of 4 hours, the medium was aspirated and replaced with 0.3 ml of medium without serum, AdCMVmTRHR (300 particles/cell) was added and the cells were incubated at 37°C. After 1 hour, 0.7 to 1.0 ml medium containing serum was added and the incubation continued for 3 to 72 hours. Infection with AdCMVmTRHR was performed in an identical manner for all cell types except that the incubation mediums were different. The mediums were: Dulbecco's modified Eagle's medium supplemented with 5% Nu-Serum for human cervical cancer HeLa cells, monkey kidney Cos-1 and CV-1 cells, and rat glioma C6 cells; Ham's F-10 medium with 15% horse serum and 2.5% fetal bovine serum for rat pituitary tumor GHY cells; Delbecco's modified Eagle's medium with 10% Nu-Serum for mouse pituitary tumor AtT-20 cells; and modified Eagle's medium with 10% fetal bovine serum for human epidermoid KB cells. None of these cell lines express TRH-Rs. Cells were studied 16 to 24 hours after infection with 300 AdCMVmTRHR particles per cell which yielded maximal TRH-R expression.

4. Transfection with pAdCMVmTRHR or pCDM8mTRHR:

pCDM8mTRHR is an expression vector in which TRH-R DNA transcription is controlled by a cytomegalovirus-1 promoter and which contains the SV-40 sequence for plasmid replication in COS-1 cells (Straub et al., 1990, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 87, pp. 9514-9518, which disclosure is hereby incorporated by reference). One of two DEAE dextran methods (Cullen, B.R., 1987, *Methods. Enzymol.*, vol. 152, pp. 684-704, which disclosure is hereby incorporated by reference) that yielded the higher level of expression was used depending on the cell type. For HeLa, CV1 and COS-1 cells, a protocol that included incubation with pAdCMVmTRHR or pCDM8mTRHR and DEAE dextran at 37°C, incubation with 0.08 mM chloroquine for 2.5 hours and addition of dimethylsulfoxide (10%) for 2.5 minutes was used (Straub et al., 1990, *Proc. Natl. Acad. Sci. U.S.A.*, vol. 87, pp.

9514-9518, which disclosure is hereby incorporated by reference). For GHY, AtT-20 and C6 cells, incubation with plasmid and DEAE dextran was for 0.5 hours at 4°C and no chloroquine or dimethylsulfoxide was added (Fujimoto et al., *Endocrinology,* vol. 130, pp. 1879-1884, which disclosure is hereby incorporated by reference). Cells were studied 48 to 72 hours after transfection, which are times of maximum TRH-R expression.

5. Measurement of TRH-R number:

Binding of 0.1 to 7.5 nM [^{3}H]*methyl*TRH, an analog of higher affinity and potency than TRH (Vale et al., 1971, *Endocrinology,* vol. 89, pp. 1485-1488, which disclosure is hereby incorporated reference), to intact cells was measured as described by Gershengorn, M.C., 1978, *J.Clin. Invest.,* vol. 62, pp. 937-943, which disclosure is hereby incorporated by reference. Binding isotherms were fitted and dissociation constants (K_ds) and receptor numbers (one-to-one stoichiometry of *methyl*TRH and receptor) were obtained with the INPLOT program (Graphpad). Receptor number was calculated using the following equation: fractional occupancy = $1 / [1 + (K_d/L)]$. Receptor number is given assuming that all cells in the population are expressing equal numbers of TRH-Rs. This appears to be the case with infections using 300 AdCMVmTRHR particles per cell (not shown).

6. Measurement of TRH response:

Infected or transfected cells were labelled for 24 hours with [^{3}H]*myo*-inositol, stimulated with TRH or *methyl*TRH in a balanced salt solution containing 10 mM LiCl and [^{3}H]IPs were measured as described by Imai et al., 1987, *Methods. Enzymol.*, vol. 141, pp. 100-101, which disclosure is hereby incorporated by reference.

7. Measurement of desensitization and inhibition by PMA:

Cells were incubated in medium with serum containing *myo*-[^{3}H]inositol (1 μCi/ml) for 24 hours prior to infection and studied 16 to 24 hours after infection.

The desensitization protocol was as described by Perlmand et al., 1991, *Endocrinology*, vol. 129, pp. 2679-2686, which disclosure is hereby incorporated by reference, except all incubations were at 37°C. Stimulation by TRH was in cells incubating in medium with serum containing *myo*-[^{3}H]inositol to prevent depletion of ^{3}H-labelled phosphoinositide substrate. The rate of IP formation was determined by linear regression analysis of the amount of [^{3}H]IPs, expressed as % of ^{3}H-labelled phosphoinositides, per minute during a 30 minute incubation. The desensitized rate is measured after 60 minutes of stimulation by 1 μM TRH by adding LiCl to a final concentration of 10 mM. The initial rate of TRH-stimulated IP formation is measured by adding TRH and LiCl simultaneously (at 60 minutes in parallel with the desensitized cells). In experiments with PMA, PMA was dissolved in dimethylsulfoxide and was added 60 minutes prior to TRH and LiCl to a final concentration of 0.1μM.

8. Internalization of TRH-Rs:

Internalization was measured as specifically bound [^{3}H]*methyl*TRH that was resistant to acid wash (Hinkle et al., 1982, *J.Biol. Chem.*, vol. 257, pp. 5462-5470; and Nussenzveig et al., 1993, *J.Biol. Chem.*, vol. 268, pp. 2389-2392, which disclosures are hereby incorporated by reference). Specific acid resistant binding was calculated by subtracting the nonspecifically bound from the [^{3}H]*methyl*TRH remaining after acid/salt elution.

RESULTS

A highly efficient, replication defective recombinant adenovirus, AdCMVmTRHR, was constructed which contains the coding sequence of the mouse TRH-R under the control of the cytomegalovirus-1 promoter and RNA processing elements inserted at the E1 region of a parent adenovirus-5 genome, dl309 or a derived derivative (Jones et al., 1979, *Cell*, vol. 17, pp. 683-689, which disclosure is hereby incorporated by reference). The strategy employed for the construction of

AdCMVmTRHR (Fig. 13) was similar to that used in the construction of the plasmid vector constructs of the invention (Figs. 1(a) & 1(b)). With reference to Figure 13, there is shown plasmid vector pAdCMVmTRHR, which was used to produce recombinant adenovirus AdCMVmTRHR. Turning to Figure 13, the left end of the adenovirus starts at position 1. The adenovirus sequence from nucleotide 1-353 contains the origin of replication and the viral packaging sequence. The adenovirus sequence from 354-2800 is deleted and replaced with the CMV-1 promoter, splice elements, the protein coding region of the mouse TRH-R cDNA sequence and the E2 poly(A) site. The left end adenovirus sequence from nucleotides 2800-5776 serve as the region for homologous recombination.

The novelty of AdCMVmTRHR as a vector for expression of TRH-Rs and its advantage over transfection are illustrated in Figure 14. With reference to Figure 14, the levels of TRH-R expression (upper panel) and *methyl*TRH stimulation of [^{3}H]IP formation (lower panel) were measured as previously described. The data in the (upper panel) are presented as number of receptors per cell assuming that all cells express equal numbers of TRH-Rs. The bars in both panels represent the mean ±SD of triplicate determinations in a representative experiment that was performed 3 times. In these experiments the plasmid vector used for virus construction, and expression of TRH-Rs after infection with AdCMVmTRHR and after transfection with pAdCMVmTRHR, were compared in HeLa cells, rat pituitary tumor GHY cells, mouse pituitary tumor AtT-20 cells, rat glioma C6 cells and monkey kidney CV1 and COS-1 cells. These cell lines were chosen because they represent a wide variety of cell types which do not express TRH-Rs. That is, HeLa cells were studied because they are readily infected with adenovirus. GHY cells were studied because they are a subclone of the cells in which endogenous TRH-Rs have been most well-studied. COS-1 cells were studied because they are a commonly used, transformed cell line that permits high levels of expression during transient assays.

TRH-Rs expressed on the surface of these cells after infection with AdCMVmTRHR bound *methyl*TRH with the same affinity as native TRH-Rs on mouse pituitary cells (Gershengorn et al., 1978, *J. Clin. Invest., vol.* 62, pp. 937-943, which

disclosure is hereby incorporated by reference) or TRH-Rs stably (Fujimoto et al., 1992, *Endocrinology*, vol. 130, pp. 1879-1884, which disclosure is hereby incorporated by reference) or transiently (Straub et al., 1990, *Proc. Natl. Acad. Sci. USA, vol.* 87, pp. 9514-9518; and Perlman et al., 1992, *J. Biol. Chem., vol.* 267, pp. 24413-24417, which disclosures are hereby incorporated by reference) expressed on several different cell types including COS-1 and HeLa cells after transfection. The dissociation constant for *methyl*TRH binding was 1.09±0.26 nM (data not shown). An important finding was that there was a higher level of TRH-R expression in every cell type except COS-1 cells when gene transfer was mediated by AdCMVmTRHR infection compared to transfection with pAdCMVmTRHR (Fig. 14, upper panel) or with plasmid, pCDM8mTRHR (Straub et al., 1990, *Proc. Natl. Acad. Sci. USA, vol.* 87, pp. 9514-9518, which disclosure is hereby incorporated by reference), that can replicate efficiently in COS-1 cells (data not shown). Under the conditions studied, there were marked differences among the various cell types in the levels of expression of TRH-Rs after infection by AdCMVmTRHR. Although the optimal conditions for AdCMVmTRHR-mediated TRH-R expression in each cell type has not been determined, these differences may be related to intrinsic characteristics of the different cell types rather than differences in conditions needed for optimal infection. For example, there may be cell-specific differences in efficiencies of adenovirus infection, perhaps related to the number of adenovirus receptors, of expression of exogenous genes in general or of TRH-R specifically, or in turnover of TRH-Rs. Infection by AdCMVmTRHR led to higher levels of TRH-R expression in a wider range of cell types than transient transfection.

A proximal step after TRH-R activation is stimulation of the formation of IP second messengers (Gershengorn et al., 1986, *Annu. Rev. Physiol.*, vol. 48, pp. 515-526; and Drummong, A. H., 1986, *J. Exp. Biol.*, vol. 124, pp. 337-358, which disclosure is hereby incorporated by reference). Therefore, *ethyl*TRH stimulation of IP formation was measured as a response to TRH-R activation. Uninfected HeLa, CHY, AtT, C6, CV1 and COS-1 cells did not respond to *methyl*TRH. In parallel with the number of TRH-Rs, there was a greater stimulation of IP formation by *methyl*TRH in

all cell types after infection by AdCMVmTRHR than after transfection. However, there was no correlation between the magnitude of the *methyl*TRH response and the number of TRH-Rs when comparing different cell types. For example, *methyl*TRH stimulation of IP formation was greater in AtT-20 cells which expressed TRH-Rs at a lower number than in HeLa cells with a greater number of TRH-Rs. One explanation for this observation may be that there are differences in post-receptor components of the signal transduction cascades within these different cell types. Another finding was that the magnitude of response to *methyl*TRH in COS-1 cells was greater after infection than after transfection even though the total number of receptors was similar. This may be because all COS-1 cells expressed a maximally effective number of TRH-Rs after AdCMVmTRHR infection, whereas only a fraction of the transfected cells were expressing maximally effective numbers of TRH-Rs because infection is more efficient than transfection.

In rat GH_3 pituitary cells naturally expressing TRH-Rs, the TRH response is rapidly desensitized (Perlman et al., 1991, *Endocrinology, vol.* 129, pp. 2679-2686, which disclosure is hereby incorporated by reference). This effect occurs prior to any decrease in the number of TRH-Rs ("down-regulation") (Gershengorn, M. C., 1978, *J. Clin. Invest., vol.* 62, pp. 937-943; and Hinkle et al., 1975, *Biochemistry, vol.* 14, pp. 3845-3851, which disclosures are hereby incorporated by reference. This response to TRH is also blunted in GH_3 cells preincubated with phorbol esters, such as PMA, which activate protein kinase C (Drummong, A. H., 1986, *J. Exp. Biol., vol.* 124, pp. 337-358, which disclosure is hereby incorporated by reference). Evidence, however, is presented that these two effects are distinct and suggested that TRH-induced desensitization is not mediated primarily by protein kinase C (Perlman et al., 1991, *Endocrinology,* vol. 129, pp. 2679-2686, which disclosure is hereby incorporate by reference). Although the molecular mechanisms of TRH-induced desensitization and of PMA-induced inhibition of the TRH response have not been elucidated, it is likely that they are mediated by receptor phosphorylation (Lefkowitz et al., 1992, *Cold Spring Harbor Symp. Quant. Biol.,* vol. 57, pp. 127-134, which disclosure is hereby incorporate by reference). Because different cell types contain

different complements of protein kinases, it was possible that TRH-induced desensitization and PMA-induced inhibition of the TRH response are cell type specific. AdCMVmTRHR infection was used to express TRH-Rs in several different cell types. Figure 15 illustrates that TRH-induced desensitization and PMA inhibition of the TRH response do not occur in all cell types. With reference to Figure 15, GHY, COS-1 (COS) and KB cells were infected with 300 AdCMVmTRHR particles per cell and TRH-induced desensitization and PMA-induced inhibition of the TRH response was measured as previously described. The data represent the mean ±SD of triplicate determinations in a representative experiment that was performed two or three times. TRH-induced desensitization and PMA-induced inhibition of the TRH response were observed in both pituitary cell types studied. In AdCMVmTRHR-infected GHY cells, the response to TRH is decreased by 49±5.2% after 60 minutes of TRH stimulation and PMA inhibits the response by 25±4.6%. Similar observations were made in AdCMVmTRHR-infected AtT-20 cells in which TRH-induced desensitization led to IP formation at a rate decreased by 41±4.7% compared to control and PMA decreased the TRH response by 37±4.0%. These effects are indistinguishable from those measured with endogenous TRH-Rs in GH_3 cells (Perlman et al., 1991, *Endocrinology,* vol. 129, pp. 2679-2686, which disclosure is hereby incorporated by reference). In contrast, in AdCMVvTRHR-infected COS-1 cells, the response to TRH did not desensitize whereas PMA inhibited the TRH response by 37±8.0%. In AdCMVmTRHR-infected KB cells, which expressed $1.16\pm0.02 \times 10^6$ TRH-Rs per cell, there was no TRH-induced desensitization (0±10%) and PMA did not inhibit the TRH response (0±10%). Thus, in a limited survey of cell lines, TRH-induced desensitization and PMA-induced inhibition of the TRH response were found only in two rodent pituitary-derived cell types, PMA-induced inhibition of TRH responsiveness but not TRH-induced desensitization was observed in monkey kidney-derived cells, and neither TRH-induced desensitization nor PMA-induced inhibition of TRH responsiveness were found in human epidermoid-derived cells. These findings support our previous suggestion that TRH-induced desensitization is not mediated by protein kinase C as TRH-induced desensitization does not occur but PMA inhibits the TRH response in COS-1 cells.

Rapid internalization is another process that many GPCRs, including TRH-Rs (Nussenzveig et al., 1993, *J. Biol. Chem.*, vol. 268, pp. 2389-2392; and Hinkle, P. M., 1989, *Ann. N. Y. Acad. Sci.*, vol. 553, pp. 176-187, which disclosures are hereby incorporated by reference), undergo after binding (Dohlman et al., 1991, *Annu. Rev. Biochem.*, vol. 60, pp. 653-688, which disclosure is hereby incorporated by reference). To determine whether TRH-R internalization is cell type specific, we measured internalization of bound *methyl*TRH in three AdCMVvTRHR-infected cell lines which displayed differences in TRH-induced desensitization or PMA-induced inhibition of the TRH response, or both. Internalization in cell lines that do and do not exhibit rapid desensitization induced by TRH was measured because it has been controversial whether these two processes are related. Figure 16 illustrates that internalization of *methyl*TRH-bound TRH-Rs was faster in AdCMVmTRHR-infected GHY cells than in COS-1 cells and KB cells but that the fraction of receptors internalized after 60 minutes was similar in all three cell types. After 60 minutes, 64±7.0%, 62±2.1%, and 71±2.6% of TRH-Rs were internalized in AdCMVmTRHR-infected GHY, COA-1 and KB cells, respectively. With reference to Figure 16, internalization of TRH-Rs was measured as previously described. The data represent mean ±SD of triplicate determinations in a representative experiment performed twice. In these three cell lines, agonist-induced internalization of TRH-Rs exhibited small kinetic differences but the extent of internalization after 60 minutes, the time at which measured desensitization were similar.

A number of aspects of GPCR biology may vary when receptors are expressed in different cell types. For example, the same GPCR may activate different signal transduction pathways when expressed in different cell types (Milligan et al., 1993, *Trends Pharmacal. Sci.*, vol. 553, pp. 176-187, which disclosure is hereby incorporated by reference). Agonist-induced desensitization, which is a process that commonly accompanies activation of GPCRs, appears to be mediated by a conserved set of intracellular regulatory proteins including protein kinases and arrestin-like proteins (Lefkowitz et al. 1993, *Adv. Second Messenger Phosphoprotein Res.*, vol. 28, pp. 1-9; and Lefkowitz, R. J., 1993, *Cell*, vol. 74, pp. 409-412, which disclosures are hereby

incorporated by reference). The data demonstrated that desensitization of TRH-Rs may occur in some cell types (GHY and AtT-20 cells) but not in others (COS-1 and KB cells). Although we have been able to show TRH-induced desensitization only in cell lines derived from the pituitary gland, it can not be concluded that TRH-R desensitization occurs only in pituitary-derived cells because only a small number of cell lines were studied. In contrast to desensitization, agonist-induced TRH-R internalization occurred in GHY, COS-1 and KB cells. This finding supports previous conclusions (Kobilka, B., 1992, *Annu. Rev. Neurosci.*, vol. 15, pp. 87-114, which disclosure is hereby incorporated by reference) that the mechanisms that mediate desensitization and internalization are distinct.

In summary, a replication defective adenovirus, AdCMVmTRHR, was constructed in accordance with the present invention and used for the high efficiency expression of TRH-Rs. Using this virus, we have been able to express TRH-Rs in a variety of mammalian cell types and study several aspects of TRH-R biology in different cell environments. We found that desensitization of the TRH response is cell type specific which occurred only in pituitary-derived cells in a limited survey of cell types whereas agonist-induced TRH-R internalization is found more generally. It was concluded that adenovirus mediated gene transfer is an excellent method for expression of TRH-Rs and suggest that this approach could be extended for expression of other cell regulatory proteins in many cell types. The versatility of adenovirus-mediated gene transfer and expression of TRH-Rs not only facilitates *in vitro* studies of TRH-R biology, but should also provide a valuable *in vivo* expression vector capable of extending TRH-R studies to animal model systems.

Example VIII

Quantitative Determination of Adenovirus-Mediated Gene Delivery to Rat Cardiac Myocytes *In Vitro* and *In Vivo*

1. Isolation and culture of Rat Cardiac Myocytes:

Primary fetal cardiac myocytes were prepared from fetal day 20 Sprague-Dawley rats (Taconic Farms) by modification of the protocol of de Carvalho et al., 1992, *Circ. Res.*, vol. 70, pp. 733-742, which disclosure is hereby incorporated by reference. Cardiac cells were preplated for 1 hour in order to remove fibroblasts. $1.8X10^6$ cells were then plated per 25mm tissue culture dishes (Corning) in heart medium (Hank's salt solution supplemented with MEM Vitamin Stock, MEM amino acids, MEM non-essential amino acids, L-Glutamine (2mM), 1% Glycine, 2% Hypoxanthine, 1% Penn-Strep, $NaHCO_3$) with 10% fetal bovine serum (Hyclone). Primary adult cardiac myocytes were prepared from the hearts of 200g female Sprague-Dawley rats (Taconic Farms) according to the protocol of White et al. 1993, *Biophys. J.*, vol. 65, pp. 196-204, which disclosure is hereby incorporated by reference. 2.4×10^5 cells were plated in heart medium per 60mm dish coated with 20 ug/ml of laminin (Boehringer Mannheim). Cells were maintained in culture at 37°C, 5% CO_2. Cell culture medium was changed every other day for the duration of the assay.

2. Virus production:

Virus plaguing and the preparation of viral stocks were performed on 293 monolayer cells as described by Tantravahi et al., 1993, *Mol. Cell. Biol.*, vol. 13, pp. 578-587, which disclosure is hereby incorporated by reference.

3. Infection of cardiac myocytes:

Forty eight hours after plating, fetal myocytes were infected with AdCMVCATgD at 0.01, 0.1, 1, 10 pfu/cell. The adult cells were infected with the same doses immediately after plating. AdCMVCATgD (10^{10} pfu/ml) was diluted in heart media without added serum. One ml of media + virus was added to each 60mm

dish. The dishes were incubated for 90 minutes at 37°C, swirling gently every 15 minutes after which 1 ml of heart media (supplemented with a final concentration of 10% fetal bovine serum) was added to each dish.

4. Immunohistochemistry:

Cells were fixed on coverslips in 3.7% formaldehyde in phosphate buffered saline (8M NaCl, 0.2M KCl, 1.44M $NaHP0_4$, KH_2P0_4, pH 7.4)(PBS) for 10 minutes at room temperature. Coverslips were then washed in PBS. Cells were blocked in 10% normal goat Serum (NGS) (Jackson Immunolabs) for 2 hours at 37°C. The coverslips were then incubated for 2 hours at 37°C with a commercially available unconjugated rabbit polyclonal antibody which recognizes CAT (5 Prime-3 Prime) at a 1:1000 dilution in PBS containing 0.1% Triton, 1% NGS. Following three 5 minutes washes in PBS, the coverslips were incubated for 1 hour at 37°C with a peroxidase conjugated goat anti-rabbit antibody (BioRad) at a 1:200 dilution in PBS containing 0.1% Triton, 1% NGS. After three 5 minutes washes in PBS, the peroxidase reaction was developed using Vectastain DAB (Vector) according to manufacturer's instructions. For tissue sections, five days post-injection, hearts were removed and the distal 1/4 of the heart was placed in 3.7% formaldehyde at 4°C overnight. The samples were embedded in a paraffin (Paraplast) according to the protocol of Ausbel et al., 1989, *Current Protocols in Molecular Biology*, Wiley, New York, which disclosure is hereby incorporated by reference. 4-10μ tissue sections were cut and placed on slides coated with 0.05% w/v poly-L-Lysine (Sigma) and dried overnight at room temperature. The sections were then ethanol dehydrated, and deparaffinized in xylenes. After rehydration, sections were placed in 0.1% Triton in phosphate buffered saline (PBS) for 5 minutes. The endogenous peroxidase activity was blocked by placing the sections in 0.3% hydrogen peroxide in methanol for 30 minutes. The antibody staining procedure was carried out as previously described. Following the peroxidase developing reaction the slides were washed in distilled water (dH_2O) and the heart sections were counterstained with hematoxylin for 12 seconds. The slides

were then washed in dH_2O and mounted with gelvatol (Airvol, Air Products and Chemicals, Inc.).

5. CAT assays from myocytes:

At each time point, infected cardiac myocytes were harvested according to the protocol of Ausbel et al., 1989, *Current Protocols in Molecular Biology*, Wiley, New York, which disclosure is hereby incorporated by reference. The amount of protein in the supernatant was measured by Bradford assay using bovine serum albumin (BSA) as the standard (BioRad). CAT assays were performed on 10 μg of total protein. When the amount of CAT activity was greater than 70% and out of the linear range, supernatants were diluted in 0.1 mg/ml BSA. CAT assays were done by TLC according to the method of Kitsis et al., 1993, in *Methods in Molecular Genetics*, ed. Adolph, K.W., Academic Press, Inc., New York, Vol. 1, pp. 374-392, which disclosure is hereby incorporated by reference, incubating for 2 hours at 37°C.

6. DNA and virus injections *in vivo*:

10 μg of CMV CAT plasmid DNA in 50μl PBS was injected into the apex of the left ventricle of 200g female Sprague Dawley rats as described by *Kitsis et. al.*, 1993, in *Methods in Molecular Genetics*, ed. Adolph, K.W., Academic Press, Inc., New York, vol. 1, pp. 374-392, which disclosure is hereby incorporated by reference. For the adenovirus injections, 6×10^6 to 6×10^8 pfu in 50 μl PBS were injected, 2×10^9 pfu were injected undiluted in a volume of 50 μl.

7. CAT assays on tissue:

At indicated times following injection, hearts were removed, rinsed in PBS and weighed. For the spatial distribution experiment the hearts were then sectioned into seven roughly equivalent slices. Each slice was then homogenized using a Tissumizer (Tekmar) in a volume of 0.5 mls buffer (1M Gly gly pH 7.8, 150mM $MgSO_4$, 500mM EGTA pH 8.0, 1M DTT) for 20 sec. For the dosage, and time course experiments the hearts were homogenized the same way but in a volume equal to 0.5g

wet tissue weight per ml of buffer. The homogenates were centrifuged for 25 minutes at 4640 xg. Supernatants were then removed, heated at 65°C, and clarified in a microfuge for 5 minutes. Supernatant volumes were measured and CAT assays were done on 5% of the lysate or on dilutions of lysate in 0.1 mg/ml BSA. Assays were done as above for 2 hours at 37°C.

RESULTS

The replication defective recombinant adenovirus, AdCMVCATgD, comprising a strong eukaryotic promoter (CMV-1) and splicing elements, has proven to be a very sensitive vector for gene expression studies in human cell lines. With reference to Figure 17, there is shown the plasmid vector, pAdCMVCATgD, which was used to produce recombinant adenovirus AdCMVCATgD. Turning to Figure 17, the left end of adenovirus (0-1 map units (m.u.)) contains the origin of replication as well as the viral packaging sequence. The adenovirus sequence from 1.0-3.8 m.u.'s was deleted and replaced with the sequence elements for the CMV-1 promoter, the bacterial CAT sequence and the mouse β^{maj} globin poly(A) site. Adenovirus sequences from 3.8-15.0 m.u.'s provides DNA sequence for homologous recombination.

Recombinant adenovirus, AdCMVCATgD, was used to characterize adenovirus mediated gene transfer into cardiac myocytes *in vitro* and *in vivo*. Figure 18(a) shows the dose response and time course of AdCMVCATgD infection into primary fetal rat cardiocytes. With reference to Figures 18(a) & 18(b), relative CAT activity refers to the percent of acetylated chloramphenicol/total chloramphenicol relative to 10μg total protein multiplied by the dilution factor of the cell lysate in order to keep the assays within the linear range. The duration of study in adult cells was shortened due to reduced cell viability regardless of the presence of adenovirus. In these studies, infection was assessed both by quantitating CAT reporter gene expression and by determining the percentage of cells expressing the CAT reporter gene by immunostaining. Because of the extremely high levels of CAT activity obtained,

dilutions of cell extracts were made to maintain assays in the linear range of the CAT assay.

CAT activity was easily detected at the earliest measured time point (4 hours), was near maximal by 48 hours, and was maintained at stable levels through the remainder of the experiment (a total of 167 hours). A dose-dependent increase was maintained over a range of hour logs of virus input throughout much of the time course. The same basic extent and level of infection and expression was found in adult cardiocytes (Figure 16(b)) when infected under similar conditions. However, the duration of study was shortened to 48 hours due to the difficulty in maintaining healthy differentiated adult cardiac myocytes in culture, independent of virus infection. Based on these assays, the sensitivity of the AdCMVCATgD CAT assay, and the levels of activity resulting from these infections, it was redacted that CAT expression could be reliably detected in as few as 10 infected cells.

At each dose of virus, the percentage of fetal cells which were expressing CAT was determined by immunostaining coverslips of infected fetal cardiocytes 18 and 48 hours post infection. Mock-infected cells show no staining, but cells infected with increasing doses of virus show a proportional increase in the number of cells infected, with 1 pfu/cell (100 particles) resulting in virtually 100% of the cells being stained (data not shown). The virus infection included both myocytes and the small proportion of nonmyocyte fibroblasts (<5%) which remained in the culture following initial myocyte purification (data not shown). Similar results were obtained with adult cardiac myocytes. At an infection of 1 pfu or greater, 100% of the rod-shaped adult myocytes stained positive with an anti-CAT antibody. This was true at both 4 and 48 hours. Myocytes which were rounded up also stained positive for CAT, and sarcomeric myosin heavy chain, and excluded trypan blue (data not shown).

Adenovirus mediated gene transfer offers advantages to transient transfection assays when using cultured myocytes. The quantitative advantages of using AdCMVCATgD *in vitro* was examined to determine whether it could be extended to *in vivo* studies. 6×10^7 pfu of AdCMVCATgD virus were injected into adult rat hearts in a volume of 50 µl. A parallel injection of 10 µg of the plasmid pAdCMVCATgD was

carried out for quantitative comparison. Five days following injection of virus or DNA, hearts were sliced into approximately seven 1.5mm sections perpendicular to the long axis of the heart. The amount of CAT activity was quantitated in each section. When either plasmid DNA or AdCMVCATgD is injected into rat heart, expression of the reporter gene is localized predominantly to the vicinity of the injection site (Figure 19). Wity reference to Figure 19, total CAT activity from DNA injected hearts in relative units = 2799 +/- 1353. Total CAT activity for adenovirus injected hearts in relative units = 117,501 +/- 15,944. The fold difference in activity was calculated based on 75 ng of CAT DNA in 6 x 10^7 pfu of virus. Each line corresponds to a different animal.

Although the virus infection proved to be at least 5000 fold more efficient than the plasmid DNA injection on the basis of input DNA, the distribution of CAT activity from both DNA and virus administration is essentially identical. The highest level of expression was observed at the area of injection with a gradient of CAT activity extending towards the base of the heart.

Given the high levels of CAT activity that were obtained from virus injection, the dose responsiveness of a range of virus from 6 x 10^6 pfu up to 2 x 10^9 pfu/injection was examined. Five days following injection, hearts were homogenized and assayed for CAT activity (Figure 20(a)). Increasing CAT activity correlated with increasing virus, although not in an entirely linear fashion. With reference to Figure 20(b), there is shown the duration of CAT activity following a single injection of 6 x 10^7 pfu of AdCMVCATgD. Animals were sacrificed and CAT activity in the left ventricle was measured 15 hours, 5 days, 12 days, 21 days, 43 days, and 55 days following injection. n=4, except for the 43 and 55 day time points, where n=2. CAT activity can be detected as early as 15 hours post infection, reaching maximal levels approximately 5 days post injection. Although CAT activity is still easily detectable 43 and 55 days following injection, expression levels are 5-6 logs lower relative to peak activity. To determine the number and type of cells in the heart which express CAT, tissue sections were stained with an anti-CAT antibody. As shown in Figure 21, a very high proportion of cells in many regions of the myocardium are expressing

CAT antigen at all doses of virus. Three doses of viral input are shown. A,B=$6X10^6$; C,D=$6X10^7$; E,F=$2X10^9$. Photographs of tissue sections were taken under Differential Interference Contrast (DIC) microscopy). A,C,E; Bar = 1mm, B,D,F; Bar = .05mm. CAT positive cells are stained brown for peroxidase reaction. All sections are counterstained with hematoxylin. In many regions, virtually 100% of myocytes stain positive. Positive cells include both myocytes and nonmyocytes, although it appears that the proportion of myocytes infected exceeds that of non-myocytes. A substantial number of inflammatory cells were seen (See Figure 21(d) & 21(e)). The nature of this inflammatory response is currently under investigation but does not appear to correlate with the amount of introduced virus. The intensity of peroxidase staining appeared to increase with increasing viral dose. It appears that the lowest dose of virus ($6X10^6$) resulted in a lower intensity of CAT antigen/cell as well as reduced number of infected cells. At higher doses of virus, both an increased number of cells and an increased amount of CAT/cell were obtained.

Cardiac myocytes appear to be ideally suited for the use of adenovirus mediated gene transfer. Transient transfection of fetal cardiocytes under optimized conditions traditionally results in 10-20% of the cells being transfected. Adenovirus can infect virtually 100% of cells and does not require the use of damaging treatments such as electroporation which generally kills a large number of the cells in the culture. Clearly, fetal cardiocytes possess viral receptors in numbers do not present a limitation to use of adenovirus vectors in rat cardiocytes. With adenovirus infection, there is no apparent effect on cell viability or morphology at the pfu ratios tested here. In addition, adenovirus infections also provide an efficient means of gene transfer into adult cells which has not been possible using conventional transfection strategies (Kirshenbaum et al., 1993, *J. Clin. Invest., vol.*, 92, pp. 381-387, which disclosure is hereby incorporated by reference). A recent report of adenovirus infection of adult rat cardiocytes (Kirshenbaum et al., 1993, *J. Clin. Invest.*, vol. 92, pp. 381-387, which disclosure is hereby incorporated by reference) reported 90% infection at a dose of 10^3 pfu/cell. Based on the results of the present invention, it is not necessary to use such a high dose of virus. Because of the efficient CAT expression system, the viral dose

required for infection of virtually all cells is in the vicinity of 1 pfu/cell (100 particles). In addition, due to the ability to accurately and reproducibly assay the reporter gene activity within the first 24 hours of infection, studies on primary cell cultures can be accomplished at times when host expression functions may not have been grossly altered, which may not be the case with more conventional transfection techniques.

As shown in Figure 21, in many regions of the heart, virtually 100% of the myocytes were infected. One question that arises is whether genes introduced by adenovirus can produce enough protein to functionally modify the phenotype or physiology of a target organ or animal. We estimate that at least 150 μg of CAT protein can be expressed in a single rat heart following administration of 2×10^9 pfu of virus, suggesting that the quantity of a foreign gene product is not likely to be a limitation.

When tissue sections were stained with an anti-CAT antibody, both the number of positive cells as well as the amount of CAT protein per cell increased with increasing virus dose. This was most apparent at the two lowest doses of virus (6×10^6 and 6×10^7 pfu). This difference was not as apparent among the three highest doses of virus, probably because of the non- quantitative nature of the peroxidase stain. The adult rat heart has been estimated to have 2×10^7 myocytes, which represent about 80% of the cells in the intact heart. If adenovirus infection *in vivo* is as efficient as it is *in vitro*, then the three highest doses of virus would theoretically result in infection of all myocytes in the heart. It is difficult to estimate the total number of positive cells because of the unknown sensitivity of the antibody in a paraffin and the variation in the staining intensity. However, we can demonstrate many regions in any one heart that appear to be 100% positive, and other regions with somewhat less CAT antigen, as well as some regions that do not show any apparent staining. Visual inspection suggests that a vastly greater number of cells is infected than when plasmid DNA is introduced by injection (Kitsis et al., 1991, *Proc. Natl. Acad. Sci. USA*, vol. 88, pp. 4138-4142, which disclosure is hereby incorporated by reference).

One of the issues currently under debate concerning the use of adenovirus as a gene transfer vector is duration of expression of introduced genes. The results

obtained in accordance with the present invention and those of Lemarchand *et. al.*, 1993, *Circ. Res.*, vol. 72, pp. 1132-1138, which disclosure is hereby incorporated by reference, demonstrate a rather transient pattern of expression. It may be that in order to generate long-term expression it will be necessary to introduce the virus into neonates, as has been suggested by Strattford-Perricaudet *et al.*, 1992, *J. Clin. Invest.*, vol. 90, pp. 626-630, which disclosure is hereby incorporated by reference. Studies are currently underway to examine the effect of various routes of infection, tissue distribution and immune response to this virus *in vivo*. However, it is apparent that adenovirus mediated gene transfer in the heart is extremely efficient and should be a very useful tool for the introduction of genes into cardiac myocytes.

It should be understood, that the foregoing embodiments are provided for purpose of illustration only and, not limitation, and that all such modifications or changes which occur to persons skilled in the art are deemed to be within the spirit and scope of the present invention.

What Is Claimed:

1. A vector for expressing a heterologous gene(s) and/or gene product(s) in a host cell, comprising, at least one insertion site for cloning a selected heterologous gene; a promoter sequence positioned upstream from said gene insertion site, said gene being under the regulatory control of said promoter; the left end replication and packaging elements of the adenovirus-5 genome positioned upstream of said promoter; a eukaryotic splice acceptor and splice donor site positioned downstream of said promoter; and a polyadenylation sequence and region for homologous recombination containing a portion of the adenovirus-5 genome positioned downstream of said insertion site.

2. The vector according to Claim 1, wherein said vector is a plasmid.

3. The vector according to Claim 1, wherein said promoter sequence is the mouse cytomegalovirus early promoter, or an effective expression promoting fragment thereof.

4. The vector according to Claim 1, wherein said polyadenylation sequence is the 3' processing site from the mouse β–globin transcription unit.

5. The vector according to Claim 1, wherein said region for homologous recombination comprises the adenovirus nucleotide sequence from 2800-5776.

6. The vector according to Claim 2, wherein said plasmid vector further comprises pML vector sequences.

7. The vector according to Claim 1, wherein said vector comprises the map as shown in Figures 1(a) or 1(b).

8. The vector according to Claim 1, wherein said vector comprises the nucleotide sequence as substantially shown in Figure 22 (Seq.Id.No.1).

9. The vector according to Claim 1, wherein said vector further comprises a separate site for insertion of a second transcription unit.

10. A method of producing a recombinant adenovirus expression vector for expression of a heterologous gene(s) and/or gene product(s) in a host cell capable of being infected by said adenovirus, comprising:

a) preparing the vector according to Claim 1;

b) co-transfecting said vector with an adenovirus-5 genome in 293 cells, under conditions which facilitate homologous recombination between said vector and adenovirus-5, thereby producing a recombinant adenovirus; and

c) isolating the recombinant adenovirus.

11. A recombinant adenovirus expression vector produced according to the method of Claim 10.

12. A host cell line or animal infected by the recombinant adenovirus expression vector according to Claim 11.

13. A unicellular host transformed by the vector according to Claim 1.

14. A method for producing a selected protein, comprising, culturing a host which has been infected with a recombinant adenovirus vector according to Claim 11.

15. A method for producing a selected protein, comprising culturing a transformed host which has been transformed with a vector according to Claim 1.

16. The vector according to Claim 1, wherein said insertion site is a cDNA insertion site.

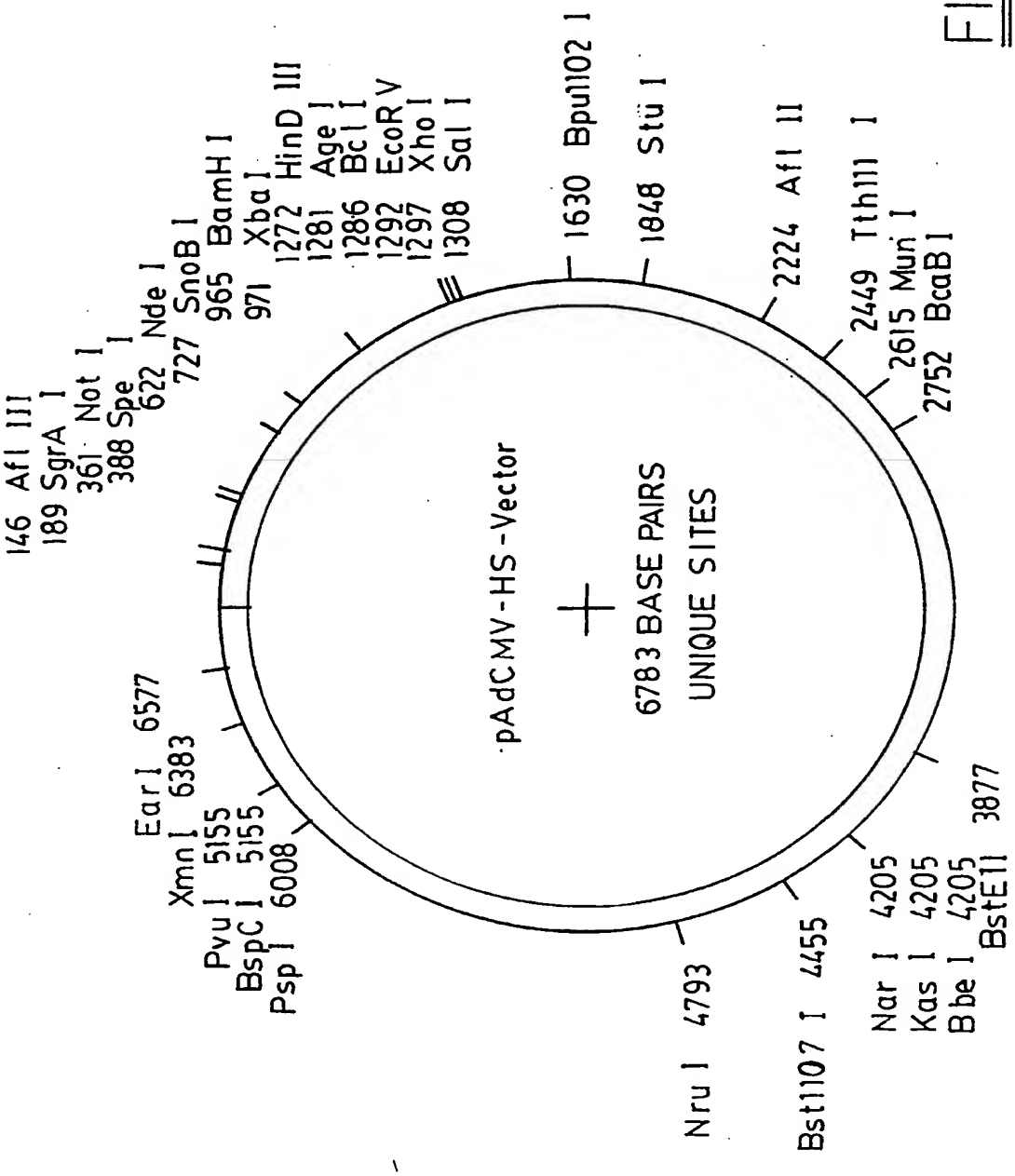

pAdCMV-HS-Vector
6783 BASE PAIRS
UNIQUE SITES
146 Afl III
189 SgrA I
361 Not I
388 Spe I
622 Nde I
727 SnoB I
965 BamH I
971 Xba I
1272 HinD III
1281 Age I
1286 Bcl I
1292 EcoR V
1297 Xho I
1308 Sal I
1630 Bpu1102 I
1848 Stü I
2224 Afl II
2449 Tth111 I
2615 Mun I
2752 BcaB I
3877 BstE11
4205 Bbe I
4205 Kas I
4205 Nar I
4455 Bst1107 I
4793 Nru I
5155 Psp I
5155 BspC I
6008 Pvu I
6383 Xmn I
6577 Ear I

FIG.1A

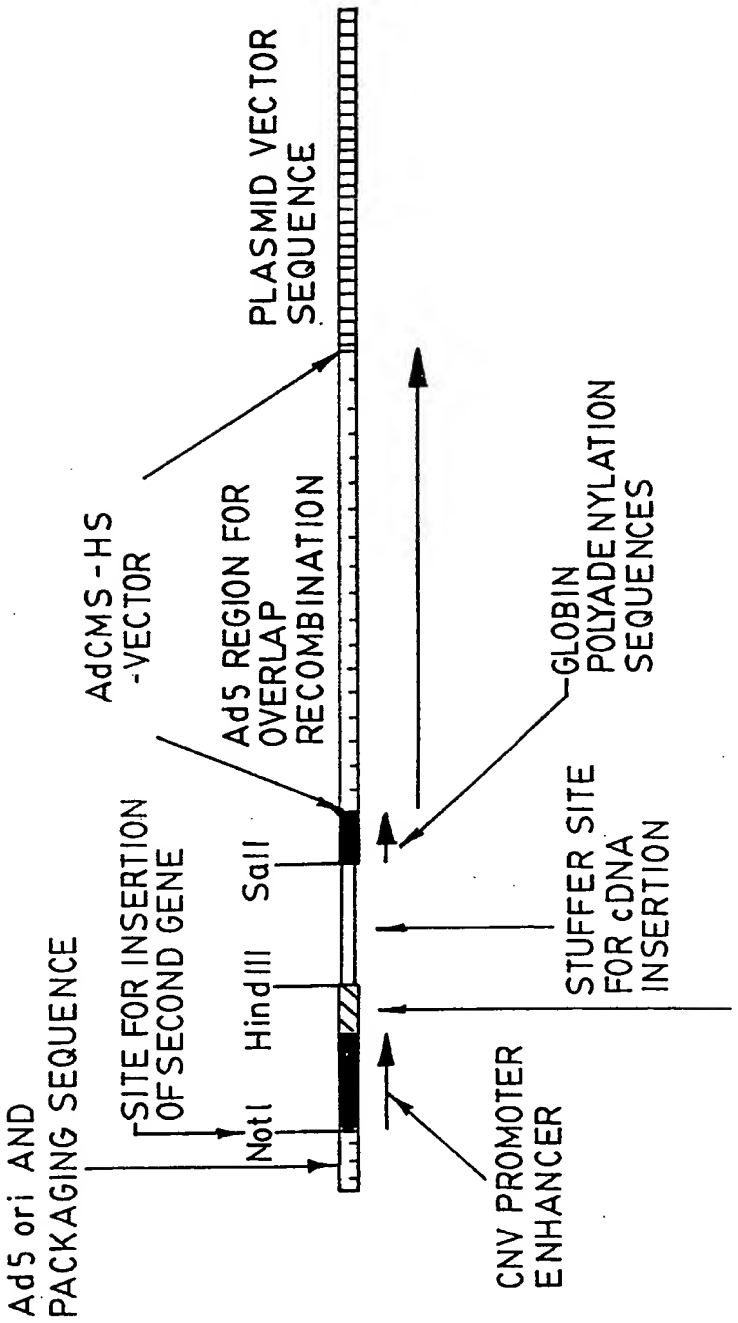
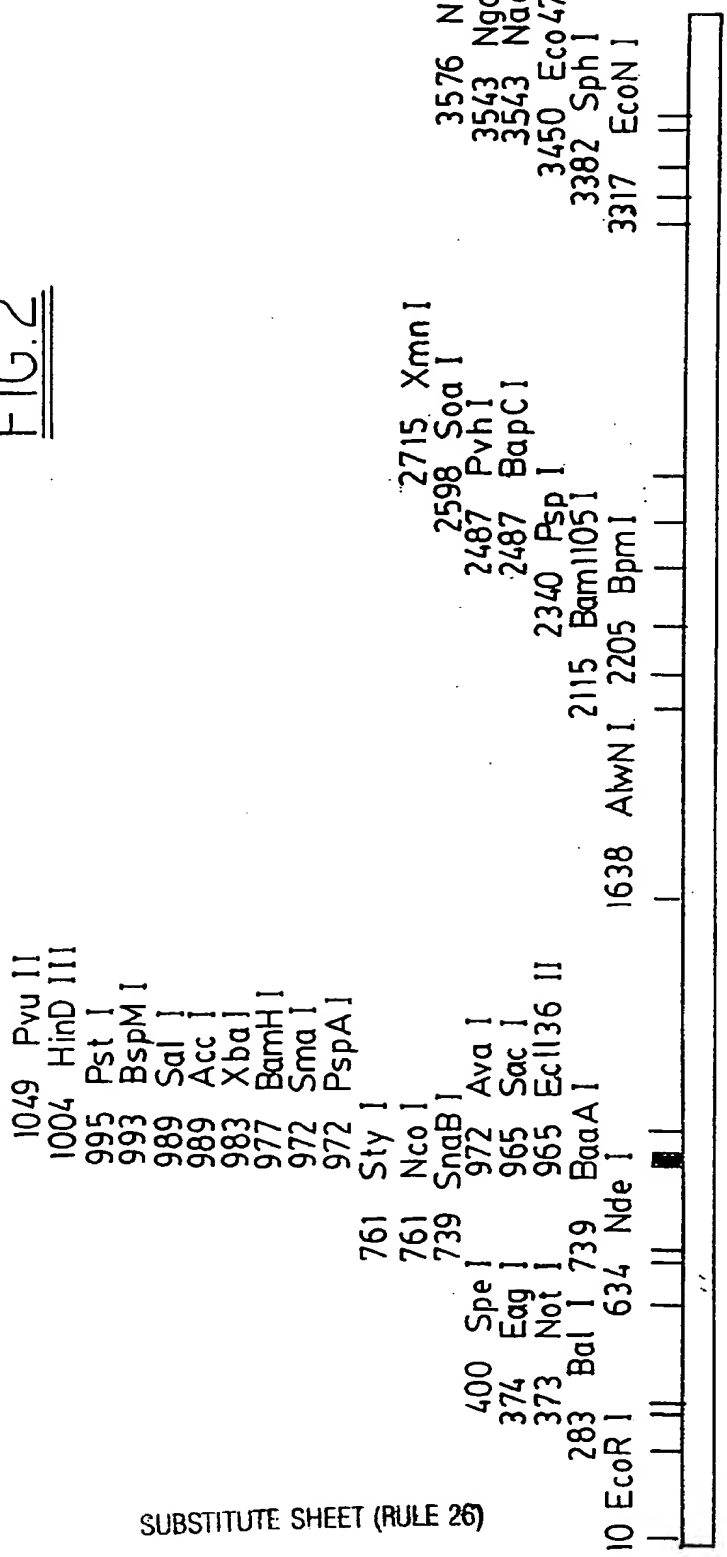

PLASMID VECTOR SEQUENCE
AdCMS-HS -VECTOR
Ad5 REGION FOR OVERLAP RECOMBINATION
GLOBIN POLYADENYLATION SEQUENCES
STUFFER SITE FOR cDNA INSERTION
SPLICE DONOR AND SPLICE ACCEPTOR
CNV PROMOTER ENHANCER
Ad5 ori AND PACKAGING SEQUENCE
SITE FOR INSERTION OF SECOND GENE
NotI
HindIII
SalI

FIG. 1B

FIG.2


pGEM2AdCMV
3818 BASE PAIRS UNIQUE SITES
10 EcoR I
283 Bal I
373 Not I
374 Eag I
400 Spe I
634 Nde I
739 BaaA I
739 Ec1136 II
761 Sac I
761 Ava I
761 SnaB I
761 Nco I
761 Sty I
972 PspA I
972 Sma I
977 BamH I
983 Xba I
989 Acc I
989 Sal I
993 BspM I
995 Pst I
1004 HinD III
1049 Pvu II
1638 AlwN I
2115 Bam1105 I
2205 Bpm I
2340 Psp I
2487 BapC I
2487 Pvh I
2598 Soa I
2715 Xmn I
3317 EcoN I
3382 Sph I
3450 Eco47 III
3543 Nae I
3543 NgoM I
3576 Nhe I

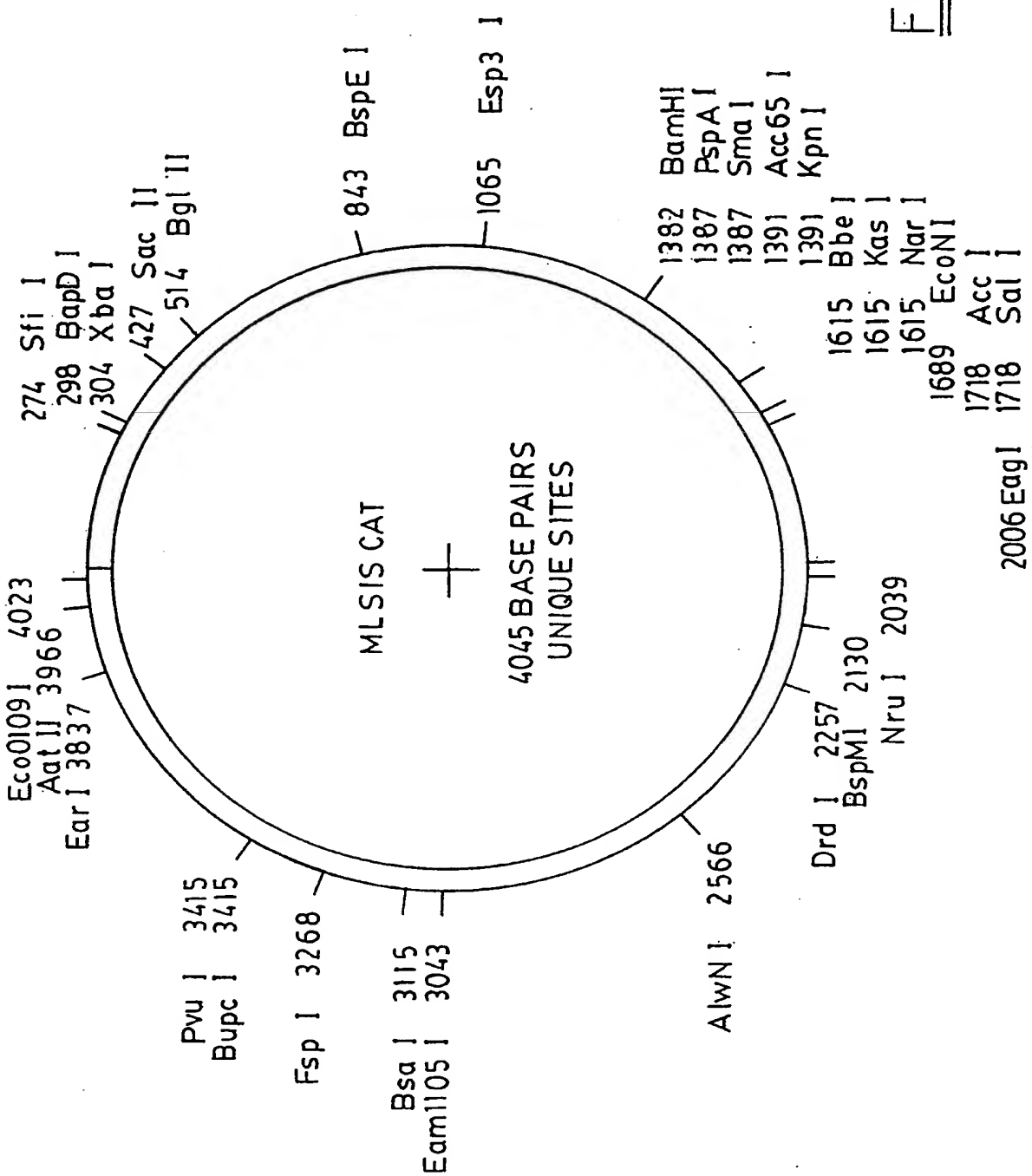

MLSIS CAT
4045 BASE PAIRS
UNIQUE SITES
Eco0109 I 4023
Aat II 3966
Ear I 3837
Pvu I 3415
Bupc I 3415
Fsp I 3268
Bsa I 3115
Eam1105 I 3043
AlwN I 2566
Drd I 2257
BspM1 2130
Nru I 2039
2006 Eag I
1718 Sal I
1718 Acc I
1689 EcoN I
1615 Nar I
1615 Kas I
1615 Bbe I
1391 Kpn I
1391 Acc65 I
1387 Sma I
1387 PspA I
1382 BamHI
1065 Esp3 I
843 BspE I
514 Bgl II
427 Sac II
304 Xba I
298 BapD I
274 Sfi I

FIG. 3

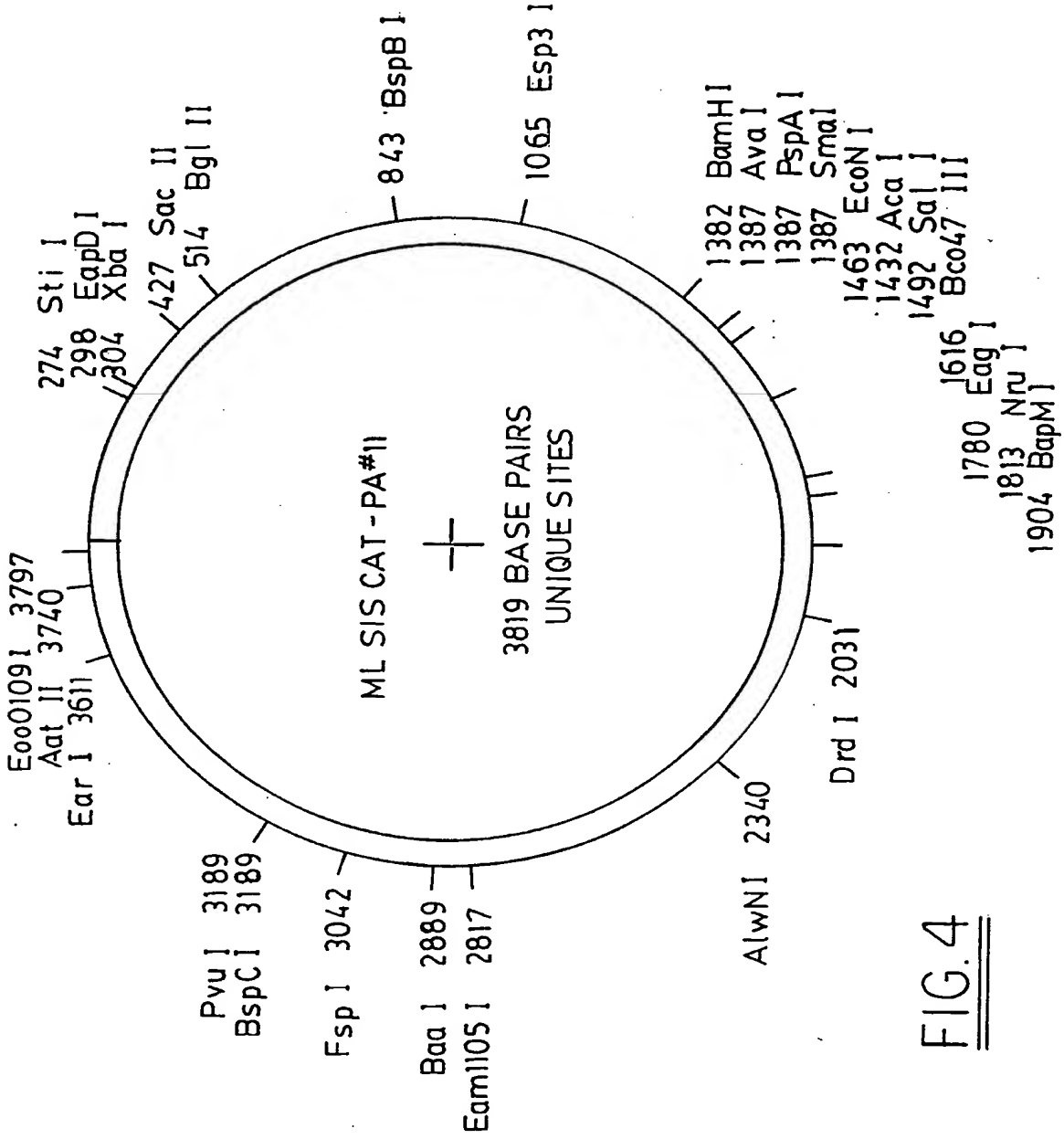

ML SIS CAT-PA#11
3819 BASE PAIRS
UNIQUE SITES
843 BspB I
1065 Esp3 I
1382 BamH I
1387 Ava I
1387 PspA I
1387 Sma I
1463 EcoN I
1432 Aca I
1492 Sal I
1616 Bco47 III
1780 Eag I
1813 Nru I
1904 BapM I
2031 Drd I
2340 AlwN I
2817 Eam1105 I
2889 Baa I
3042 Fsp I
3189 Pvu I
3189 BspC I
3611 Ear I
3740 Aat II
3797 Eco0109 I
274 Sti I
298 EapD I
304 Xba I
427 Sac II
514 Bgl II

FIG. 4

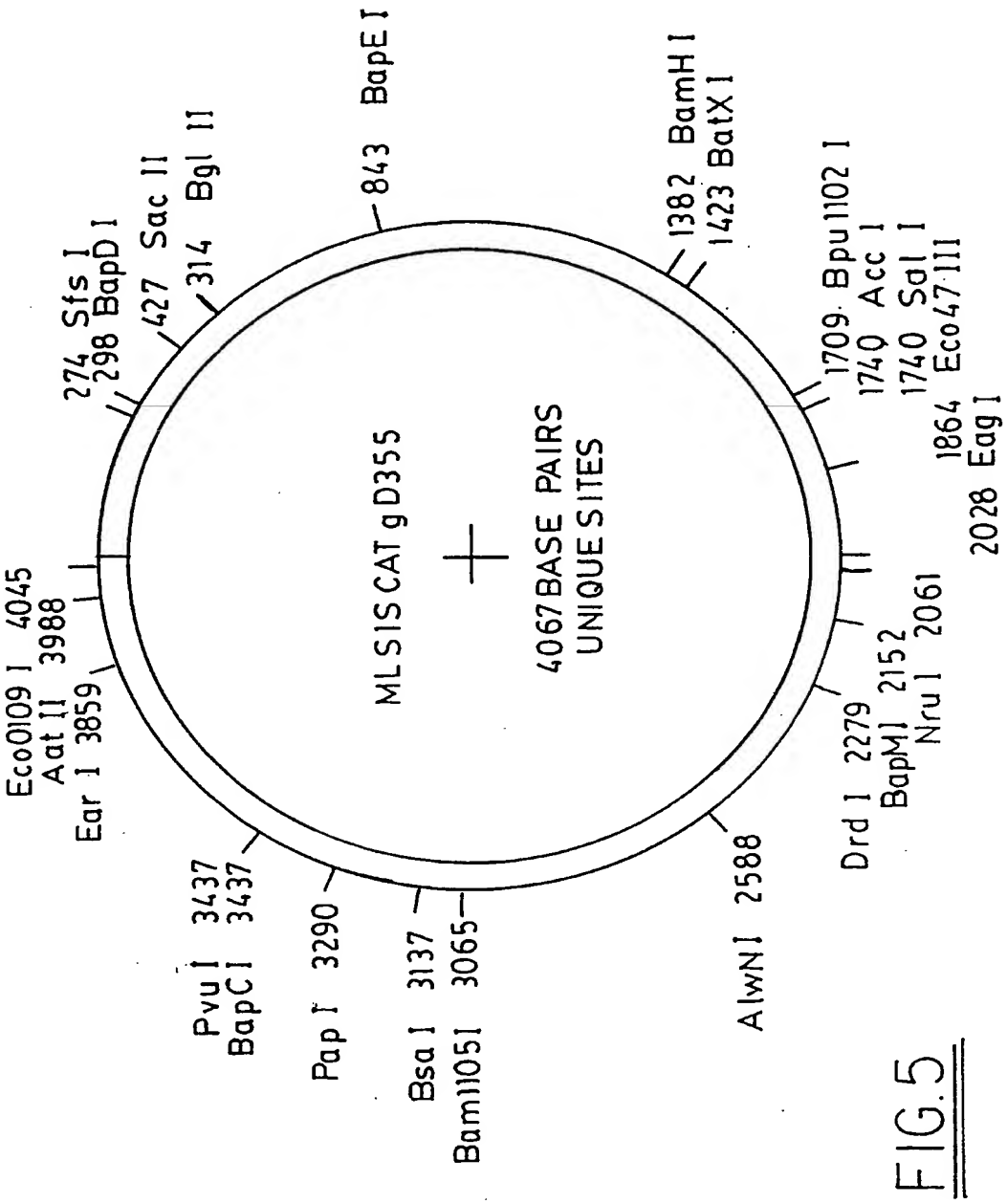

ML S1S CAT gD355
4067 BASE PAIRS
UNIQUE SITES
843 BapE I
1382 BamH I
1423 BatX I
1709 Bpu1102 I
1740 Acc I
1740 Sal I
1864 Eco47 III
2028 Eag I
2061
2152
BapMI
Nru I
2279
Drd I
2588
AlwN I
3065
3137
Bsa I
Bam11051
3290
Pap I
3437
3437
Pvu I
BapC I
3859
Ear I
3988
Aat II
4045
Eco0109 I
274 Sfs I
298 BapD I
314 Bgl II
427 Sac II

FIG.5

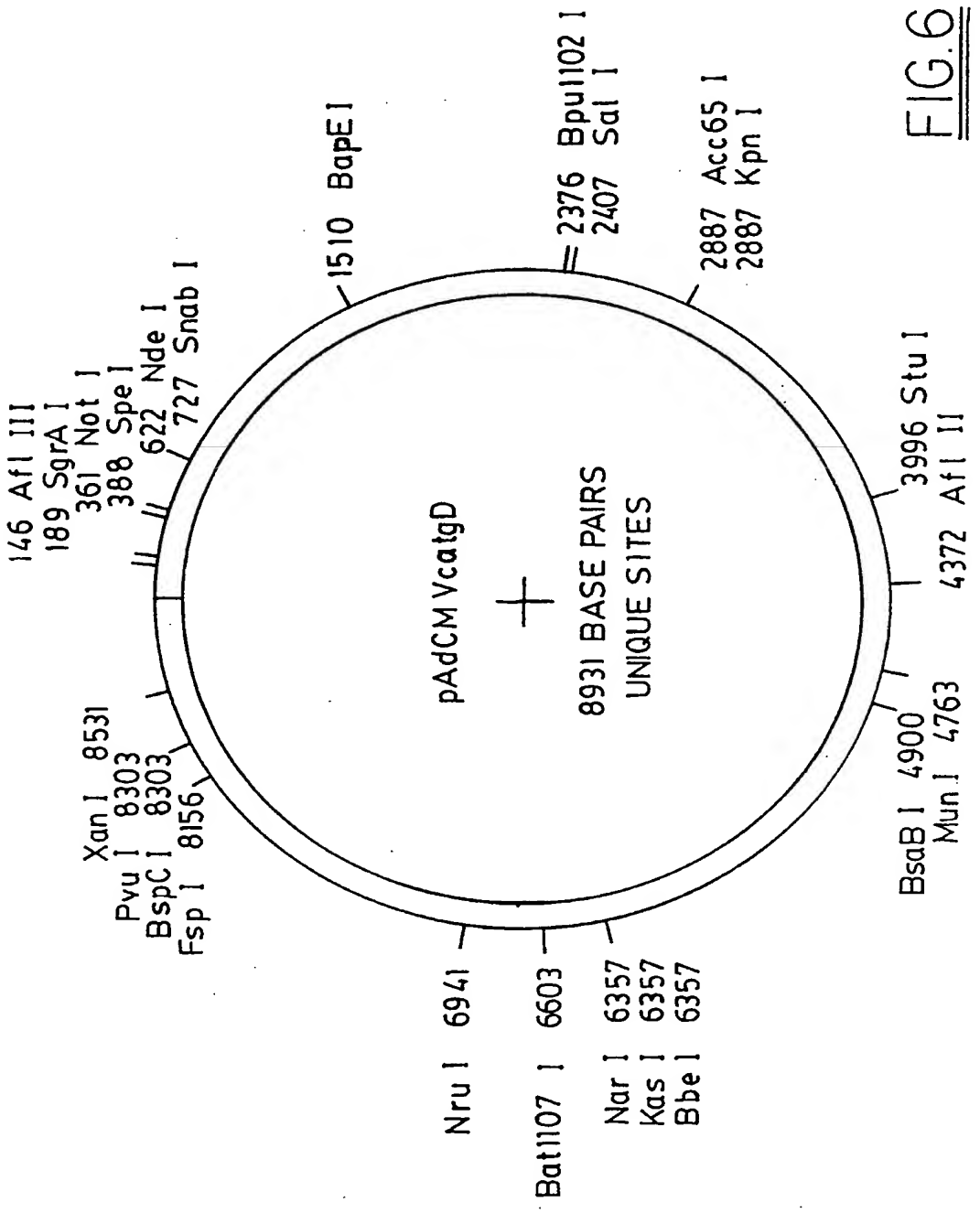
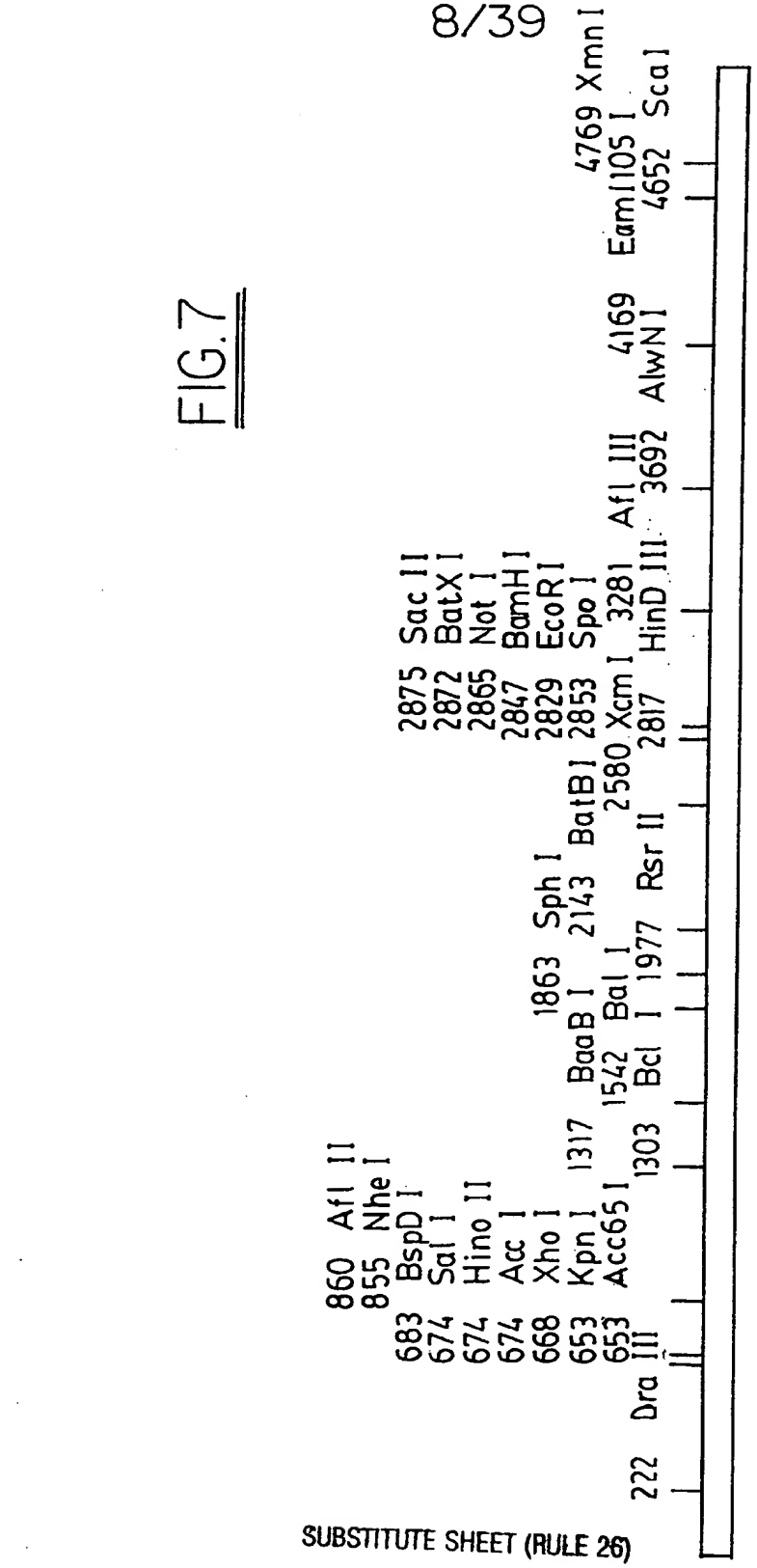

pAdCM VcatgD
8931 BASE PAIRS
UNIQUE SITES
146 Afl III
189 SgrA I
361 Not I
388 Spe I
622 Nde I
727 Snab I
1510 BapE I
2376 Bpu1102 I
2407 Sal I
2887 Acc65 I
2887 Kpn I
3996 Stu I
4372 Afl II
BsaB I 4900
Mun I 4763
Bat1107 I 6603
Nru I 6941
Nar I 6357
Kas I 6357
Bbe I 6357
Xan I 8531
Pvu I 8303
BspC I 8303
Fsp I 8156

FIG.6

FIG.7


860 Afl II
855 Nhe I
683 BspD I
674 Sal I
674 Hino II
674 Acc I
668 Xho I
653 Kpn I
653 Acc65 I
222 Dra III
1317 BaaB I
1542 Bal I
1303 Bcl I
1977 Rsr II
1863 Sph I
2143 BatB I
2580 Xcm I
2817 HinD III
2875 Sac II
2872 BatX I
2865 Not I
2847 BamH I
2829 EcoR I
2853 Spo I
3281 Afl III
3692 AlwN I
4169
4769 Xmn I
Eam1105 I
4652 Sca I
MARKSPOLYOMANEOMYCIN
5092 BASE PAIRS UNIQUE SITES

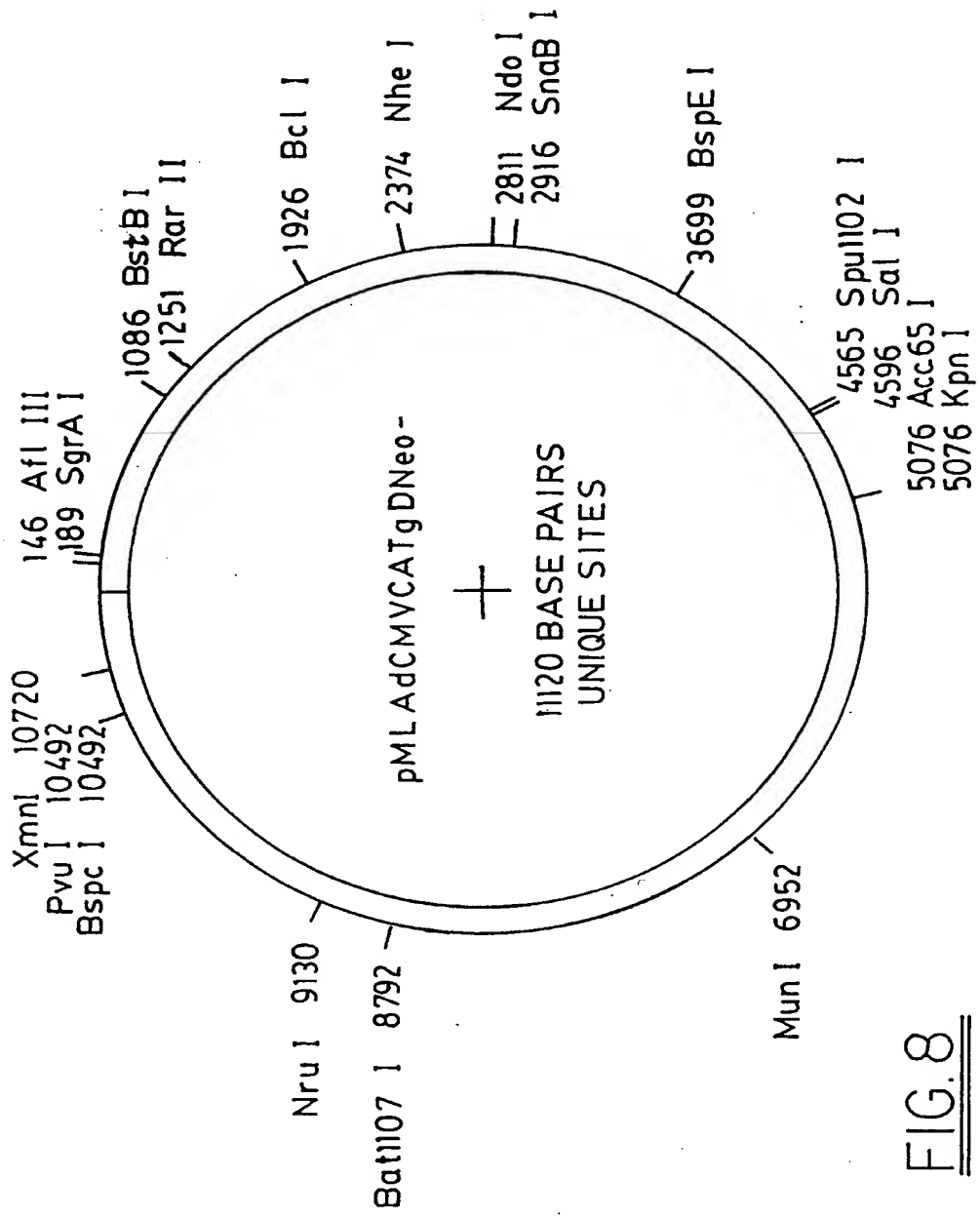

pMLAdCMVCATgDNeo-
11120 BASE PAIRS
UNIQUE SITES
Xmn I 10720
Pvu I 10492
Bspc I 10492
146 Afl III
189 SgrA I
1086 BstB I
1251 Rar II
1926 Bcl I
2374 Nhe I
2811 Ndo I
2916 SnaB I
3699 BspE I
4565 Spu1102 I
4596 Sal I
5076 Acc65 I
5076 Kpn I
Mun I 6952
Bat1107 I 8792
Nru I 9130

FIG. 8

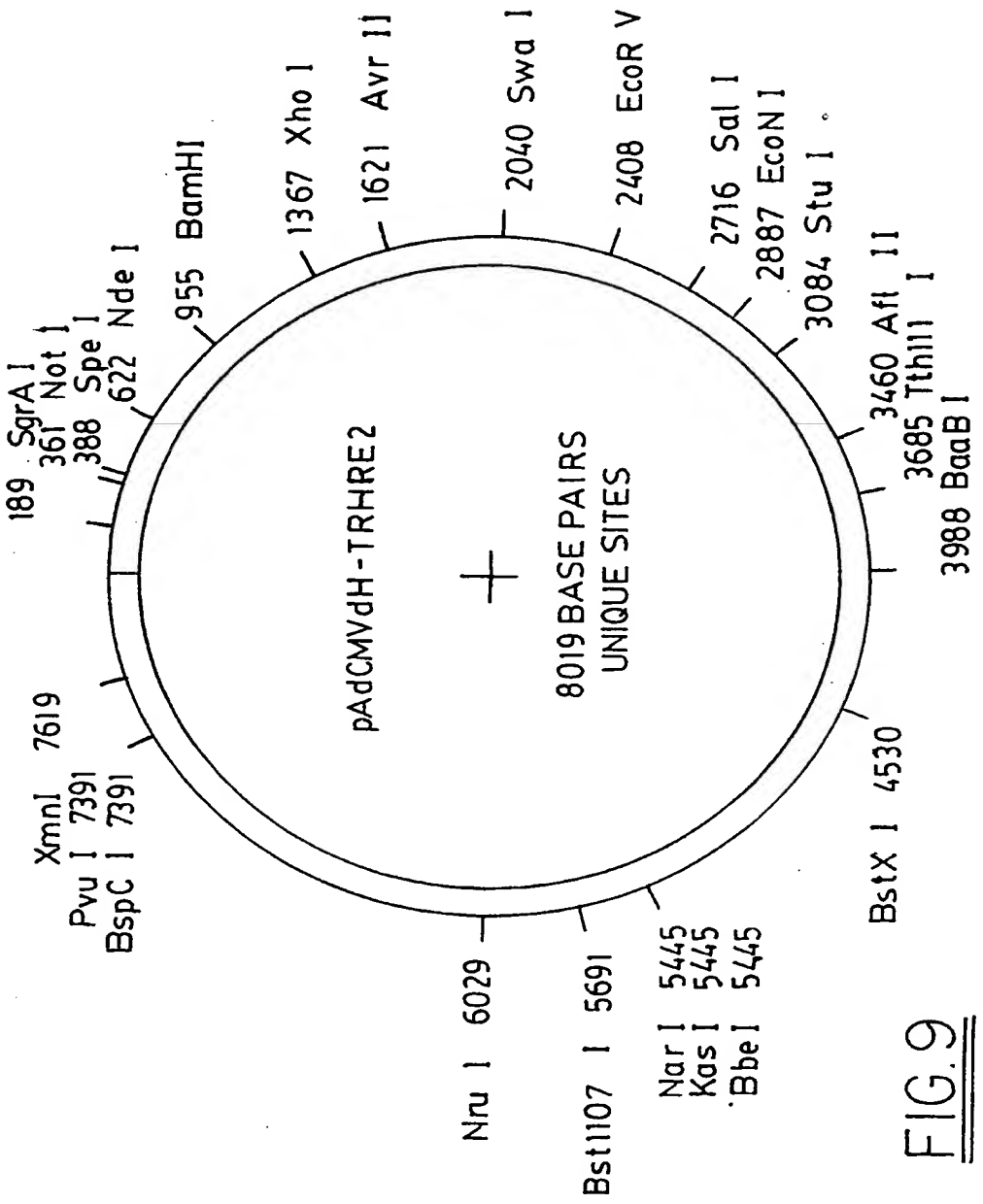

pAdCMVdH-TRHRE2
8019 BASE PAIRS
UNIQUE SITES
189 SgrA I
361 Not I
388 Spe I
622 Nde I
955 BamHI
1367 Xho I
1621 Avr II
2040 Swa I
2408 EcoR V
2716 Sal I
2887 EcoN I
3084 Stu I
3460 Afl II
3685 TthIII I
3988 BaaB I
BstX I 4530
Nru I 6029
Bst1107 I 5691
Nar I 5445
Kas I 5445
Bbe I 5445
XmnI 7619
Pvu I 7391
BspC I 7391

FIG. 9

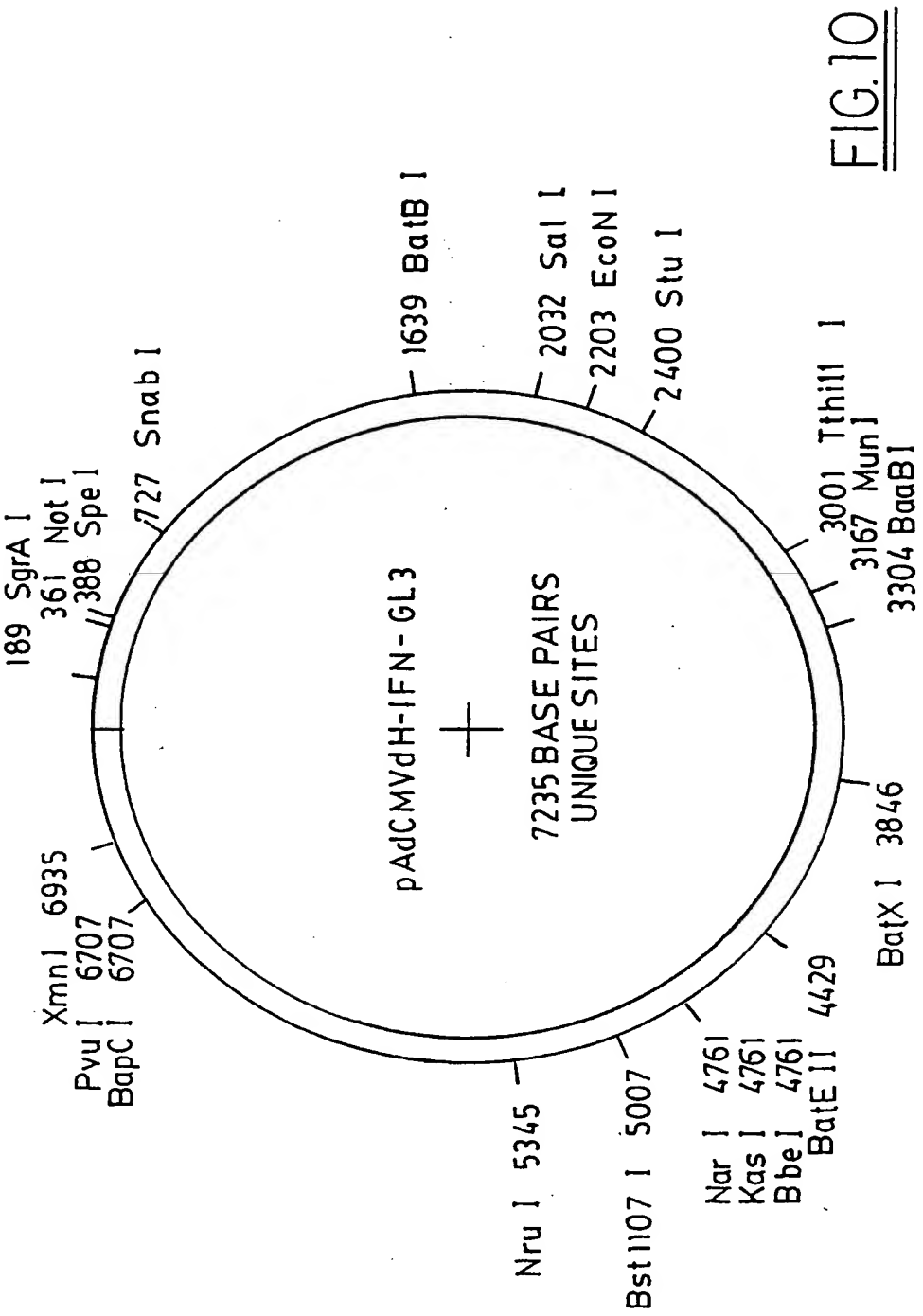

pAdCMVdH-IFN-GL3
7235 BASE PAIRS
UNIQUE SITES
189 SgrA I
361 Not I
388 Spe I
727 Snab I
1639 BatB I
2032 Sal I
2203 EcoN I
2400 Stu I
3001 Tthi11 I
3167 Mun I
3304 BaaB I
BatX I 3846
Nar I 4761
Kas I 4761
Bbe I 4761
BatE 11 4429
Bst1107 I 5007
Nru I 5345
Pvu I 6707
BapC I 6707
Xmn I 6935

FIG.10

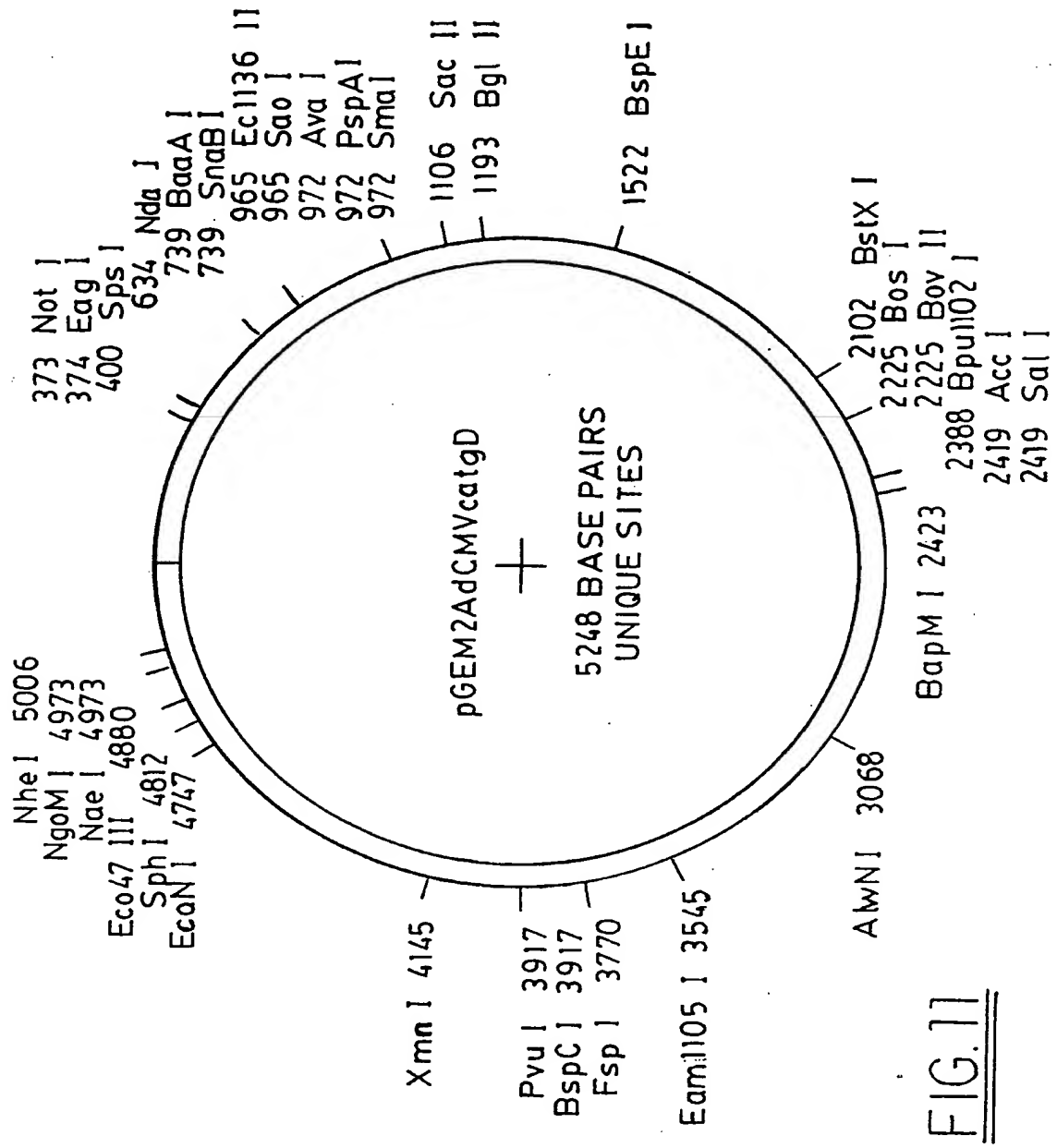

pGEM2AdCMVcatgD
5248 BASE PAIRS
UNIQUE SITES
373 Not I
374 Eag I
400 Sps I
634 Nda I
739 BaaA I
739 SnaB I
965 Ec1136 II
965 Sac I
972 Ava I
972 PspA I
972 Sma I
1106 Sac II
1193 Bgl II
1522 BspE I
2102 BstX I
2225 Bos I
2225 Bov II
2388 Bpu1102 I
2419 Acc I
2419 Sal I
BapM I 2423
AwN I 3068
Eam1105 I 3545
Pvu I 3917
BspC I 3917
Fsp I 3770
Xmn I 4145
Nhe I 5006
NgoM I 4973
Nae I 4973
Eco47 III 4880
Sph I 4812
EcoN I 4747

FIG. 11

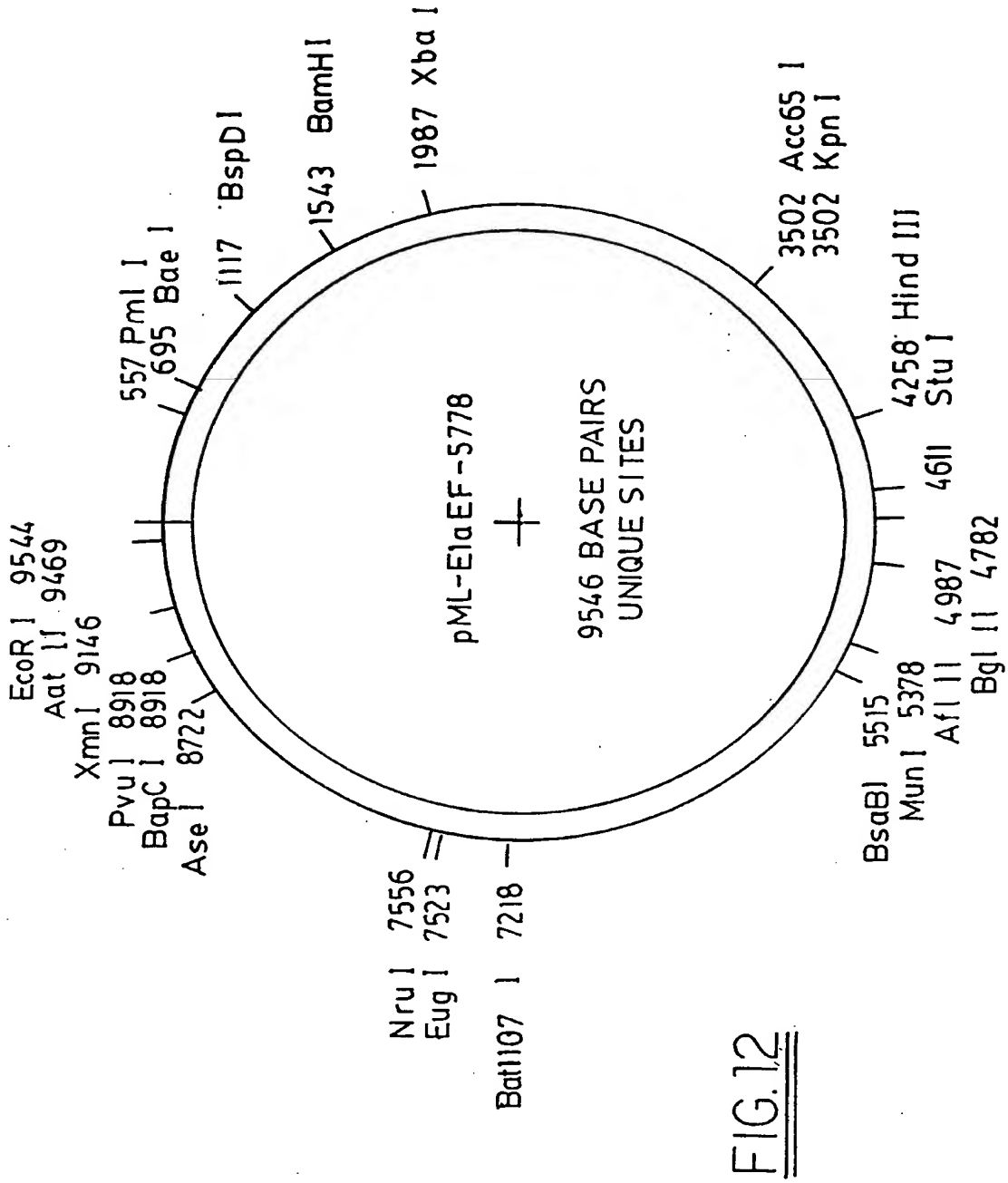

pML-E1aEF-5778
9546 BASE PAIRS
UNIQUE SITES
1987 Xba I
1543 BamHI
1117 BspDI
557 Pml I
695 Bae I
EcoR I 9544
Aat II 9469
Xmn I 9146
Pvu I 8918
BapC I 8918
Ase I 8722
Nru I 7556
Eug I 7523
Bat1107 I 7218
BsaBI 5515
Mun I 5378
Afl II 4987
Bgl II 4782
4611
4258 Hind III
4258 Stu I
3502 Acc65 I
3502 Kpn I

FIG. 12

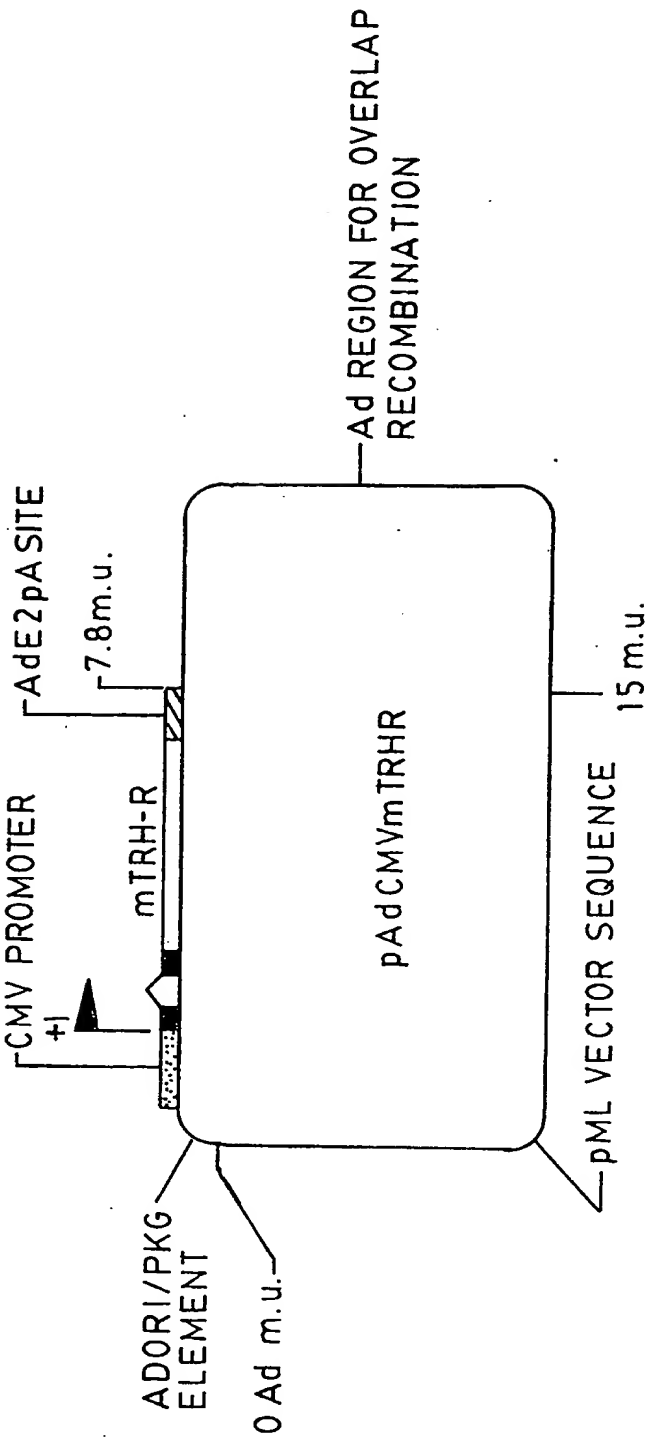

Ad REGION FOR OVERLAP RECOMBINATION
AdE2pA SITE
7.8m.u.
CMV PROMOTER
+1
mTRH-R
pAdCMVmTRHR
15 m.u.
pML VECTOR SEQUENCE
ADORI/PKG ELEMENT
0 Ad m.u.

FIG. 13

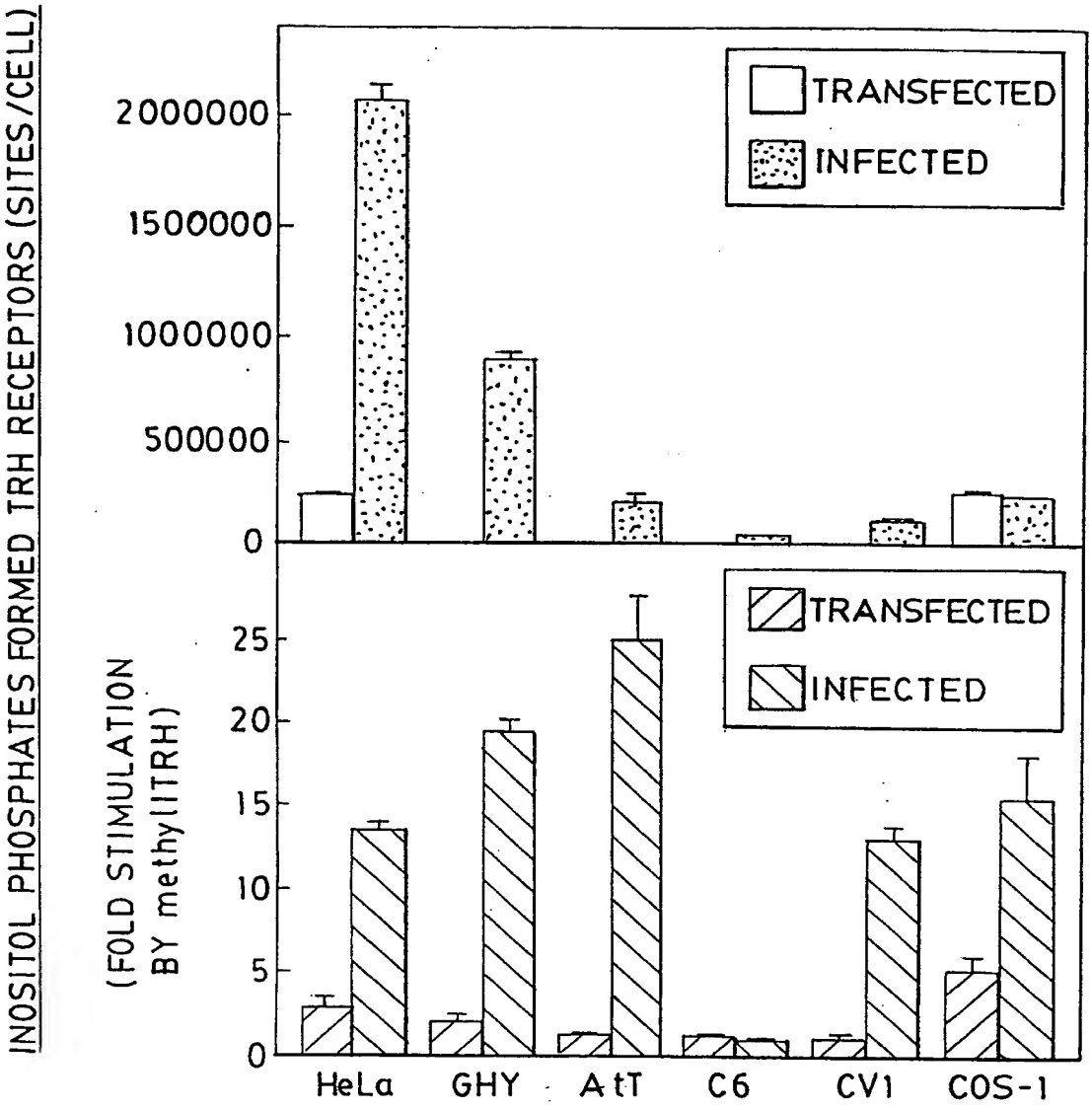

INOSITOL PHOSPHATES FORMED TRH RECEPTORS (SITES/CELL)
2000000
1500000
1000000
500000
0
TRANSFECTED
INFECTED
(FOLD STIMULATION BY methyl(ITRH)
25
20
15
10
5
0
TRANSFECTED
INFECTED
HeLa
GHY
AtT
C6
CV1
COS-1

FIG.14

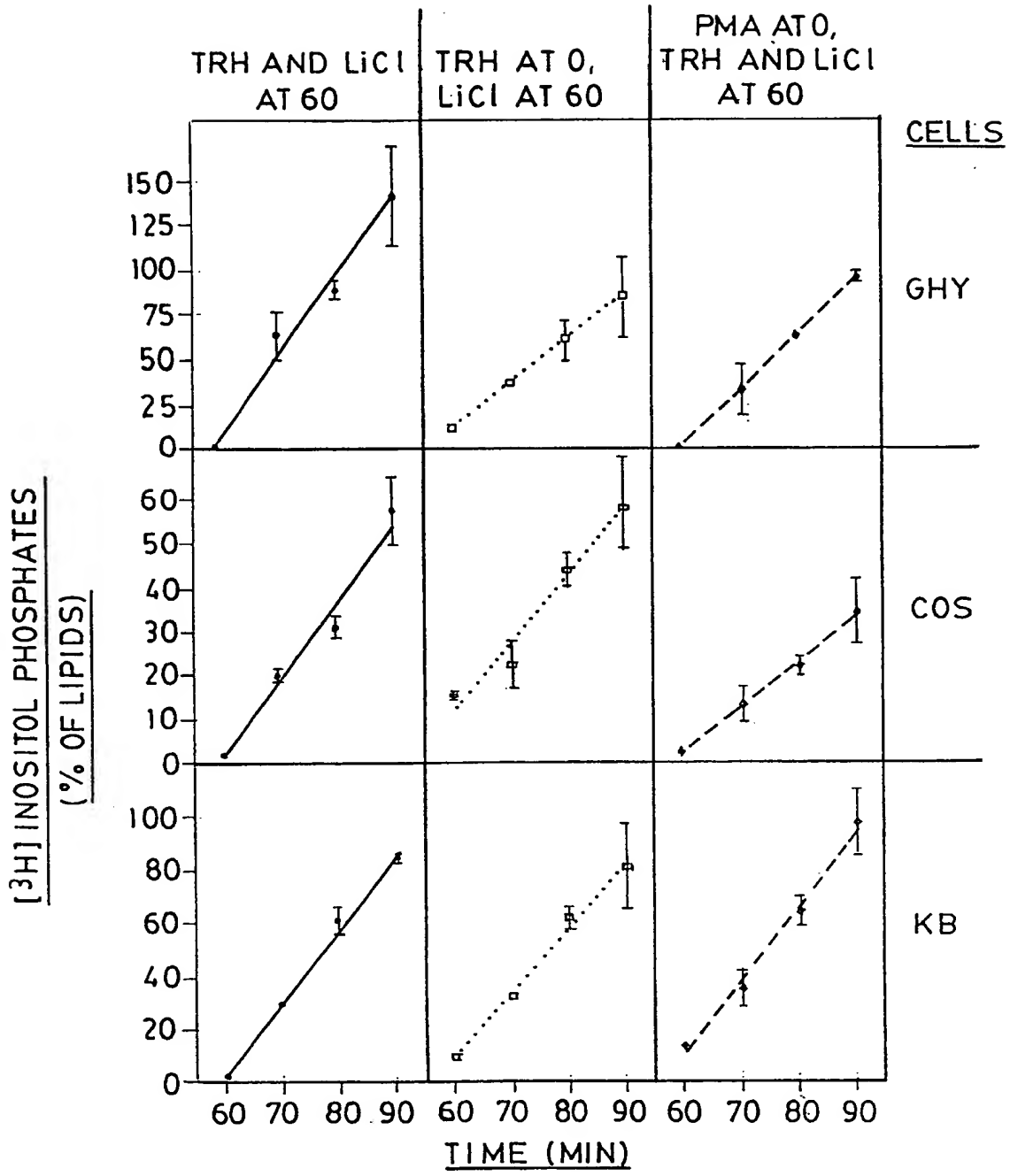

TRH AND LiCl AT 60
TRH AT 0, LiCl AT 60
PMA AT 0, TRH AND LiCl AT 60
CELLS
GHY
COS
KB
[3H] INOSITOL PHOSPHATES (% OF LIPIDS)
150
125
100
75
50
25
0
60
50
40
30
20
10
0
100
80
60
40
20
0
60 70 80 90 60 70 80 90 60 70 80 90
TIME (MIN)

FIG. 15

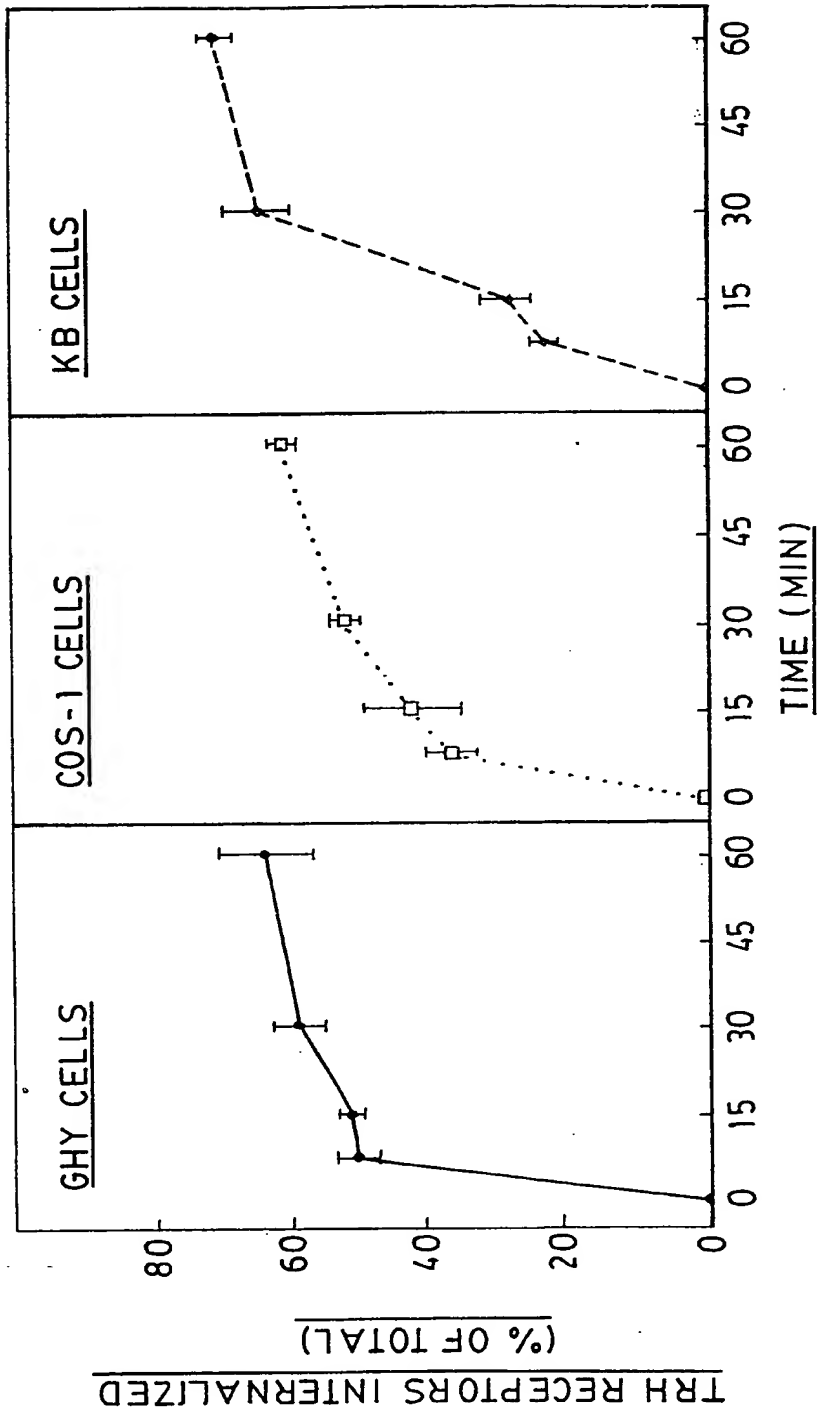

KB CELLS
COS-1 CELLS
GHY CELLS
0
15
30
45
60
TIME (MIN)
0
20
40
60
80
TRH RECEPTORS INTERNALIZED
(% OF TOTAL)

FIG.16

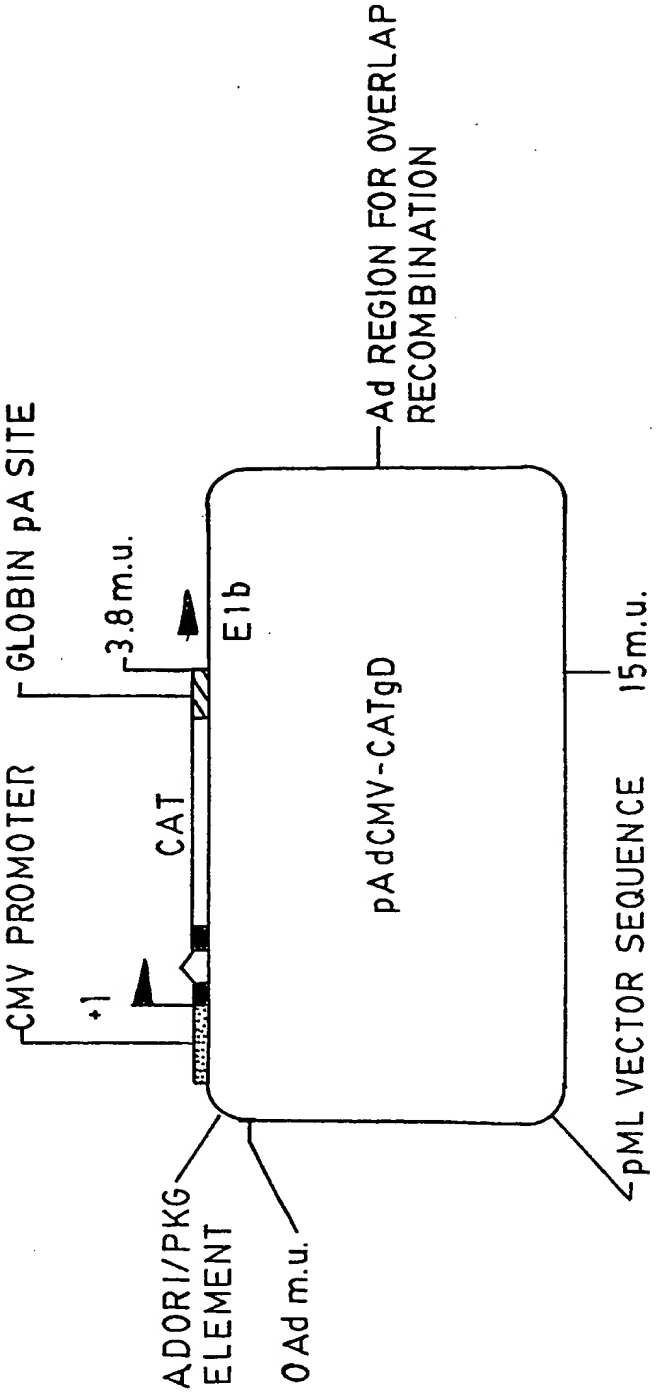

Ad REGION FOR OVERLAP RECOMBINATION
GLOBIN pA SITE
3.8m.u.
E1b
CMV PROMOTER
+1
CAT
pAdCMV-CATgD
15m.u.
pML VECTOR SEQUENCE
ADORI/PKG ELEMENT
0Ad m.u.

FIG.17

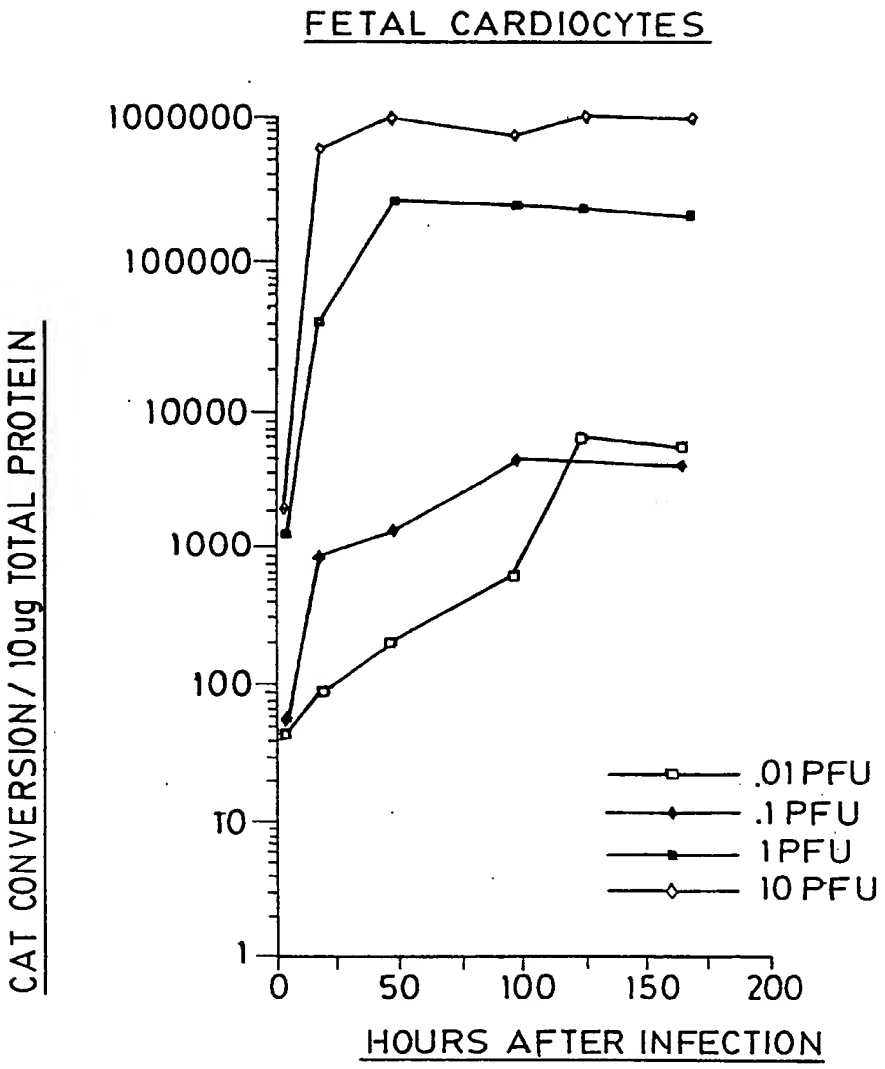

FETAL CARDIOCYTES
CAT CONVERSION/ 10ug TOTAL PROTEIN
1000000
100000
10000
1000
100
10
1
0
50
100
150
200
HOURS AFTER INFECTION
.01PFU
.1PFU
1PFU
10PFU

FIG. 18A

ADULT CARDIOSYTES

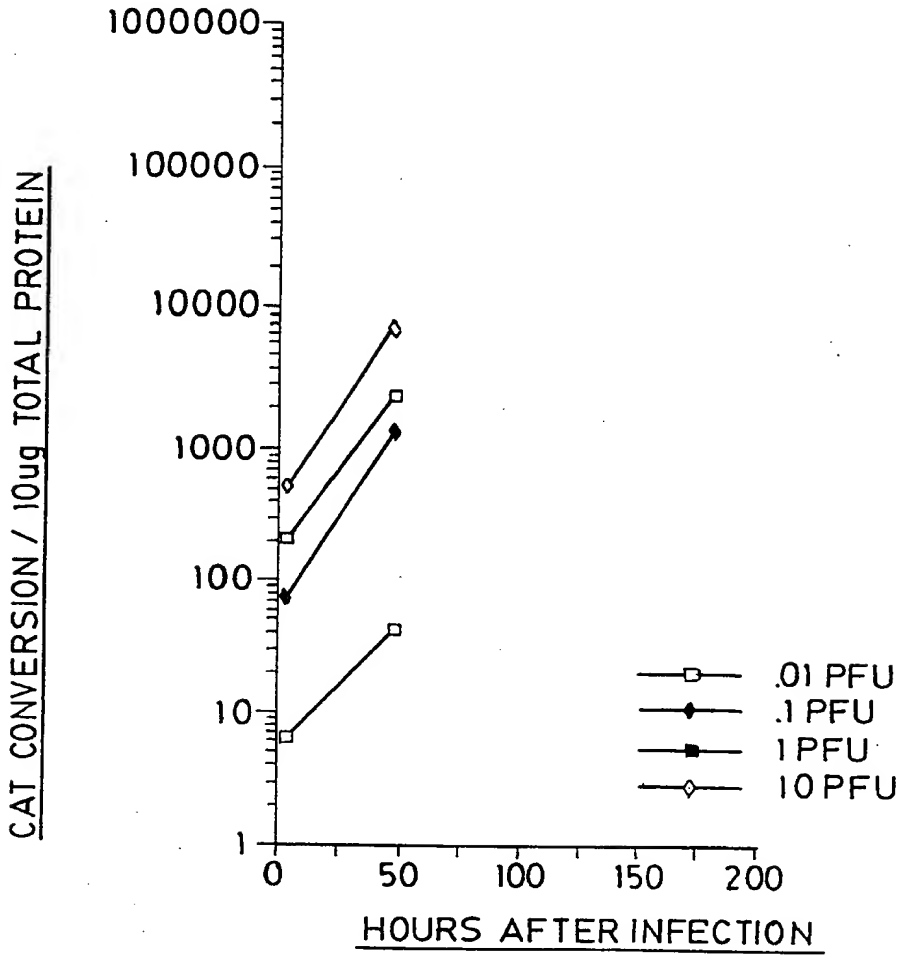


FIG. 18B

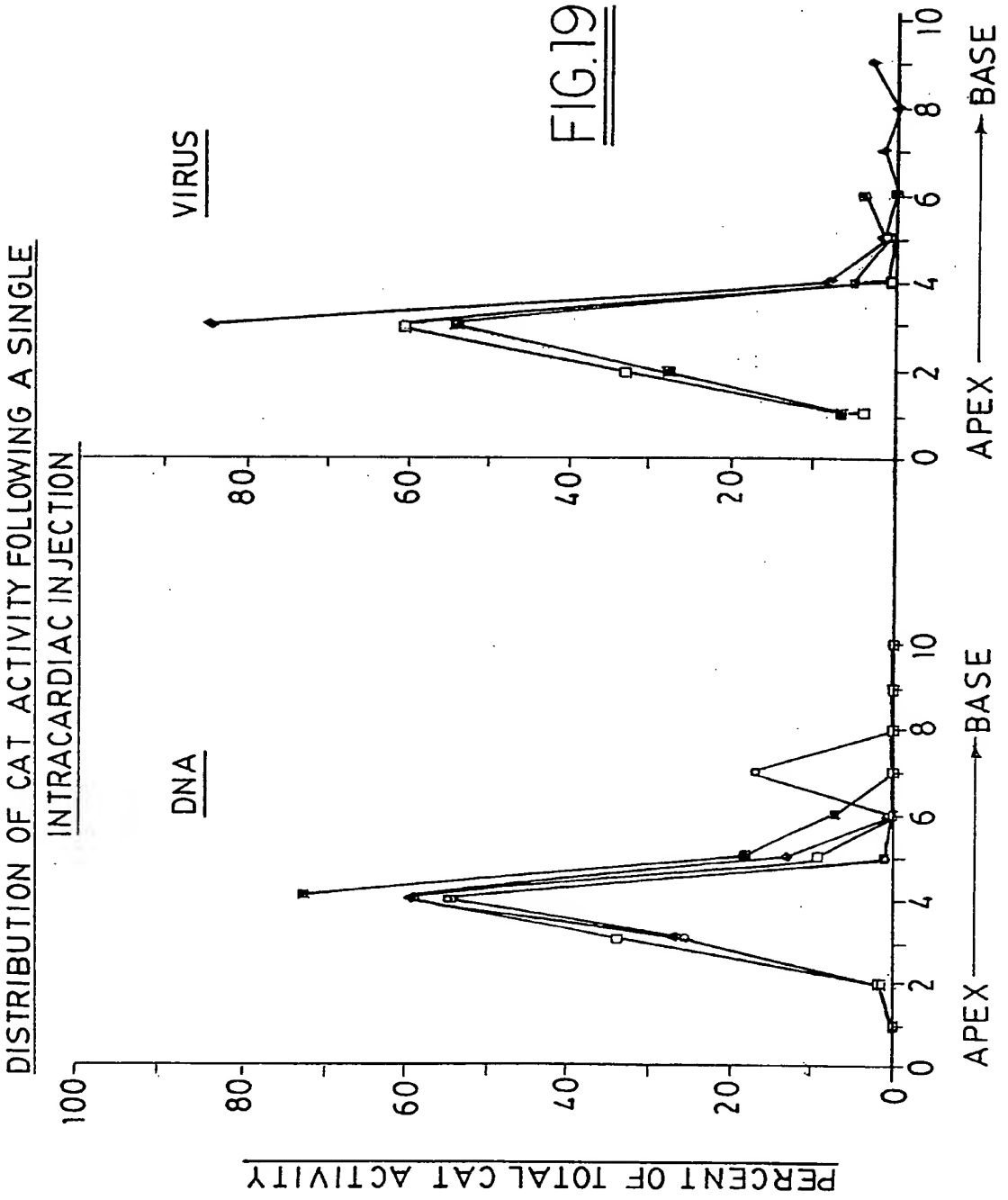

DISTRIBUTION OF CAT ACTIVITY FOLLOWING A SINGLE INTRACARDIAC INJECTION
DNA
VIRUS
PERCENT OF TOTAL CAT ACTIVITY
100
80
60
40
20
0
0
2
4
6
8
10
APEX
BASE

FIG.19

FIG 20A

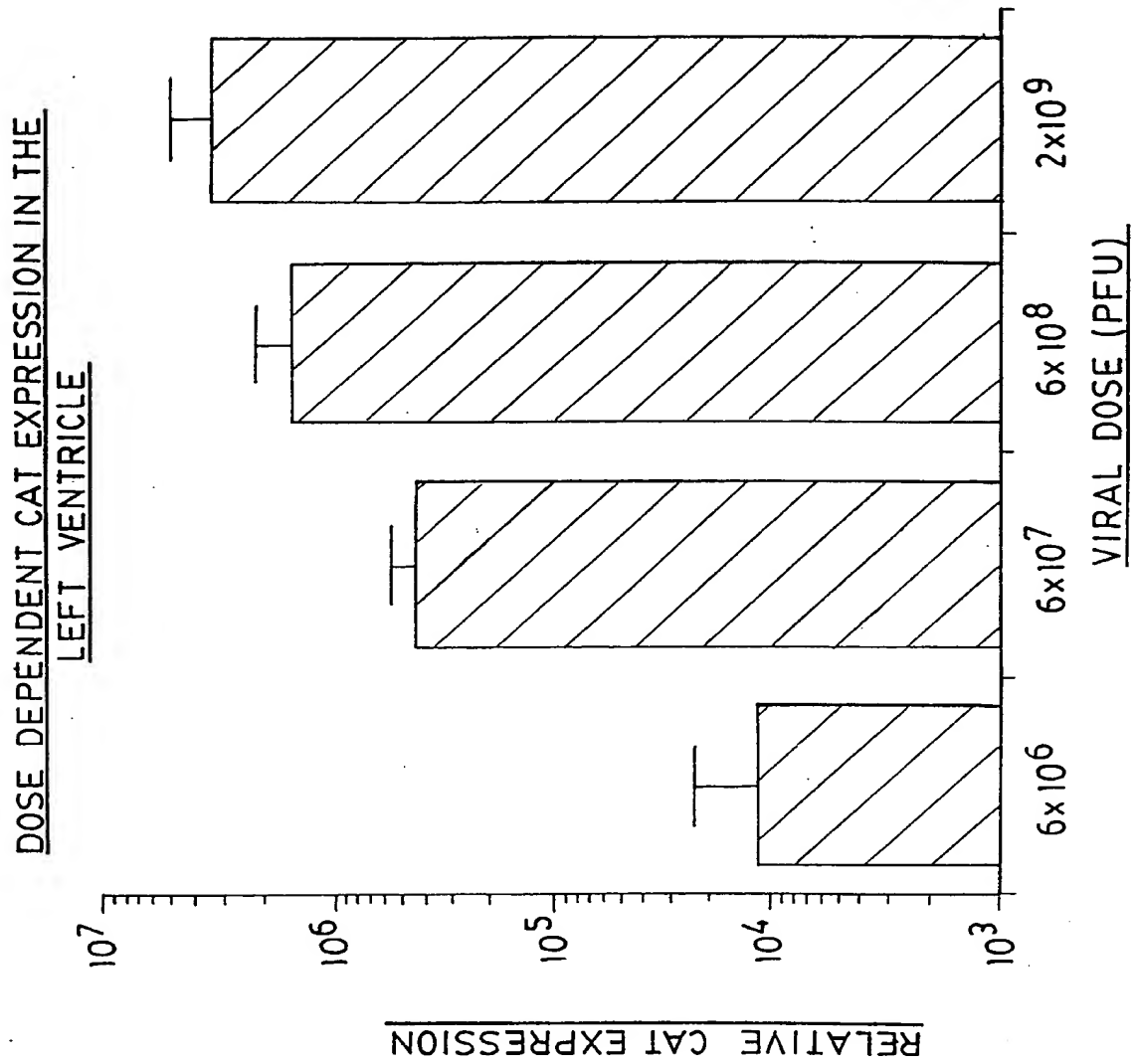
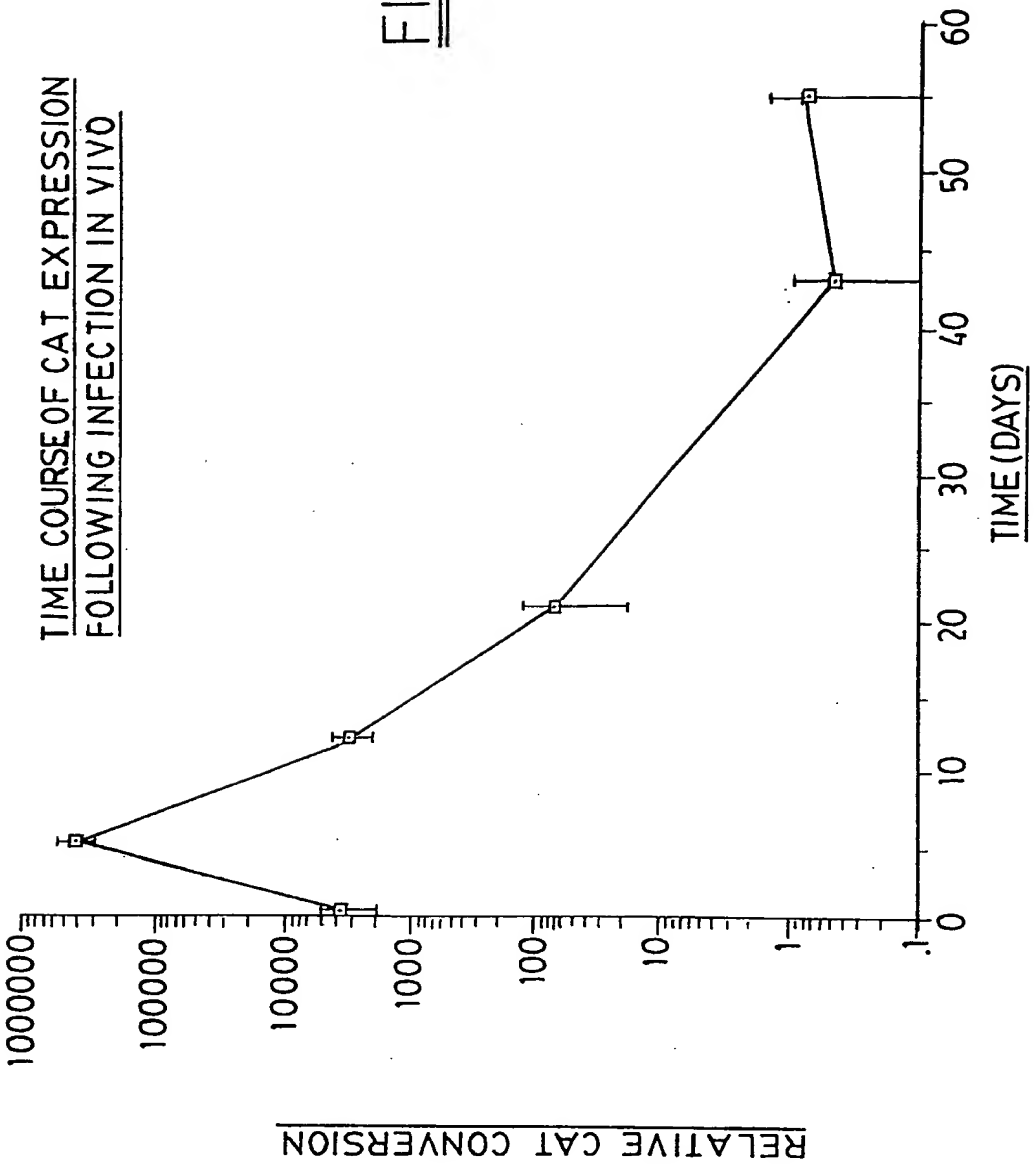

DOSE DEPENDENT CAT EXPRESSION IN THE LEFT VENTRICLE
RELATIVE CAT EXPRESSION
10^7
10^6
10^5
10^4
10^3
$6x10^6$
$6x10^7$
$6x10^8$
$2x10^9$
VIRAL DOSE (PFU)

FIG. 20B


TIME COURSE OF CAT EXPRESSION
FOLLOWING INFECTION IN VIVO
RELATIVE CAT CONVERSION
1000000
100000
10000
1000
100
10
1
.1
0
10
20
30
40
50
60
TIME (DAYS)

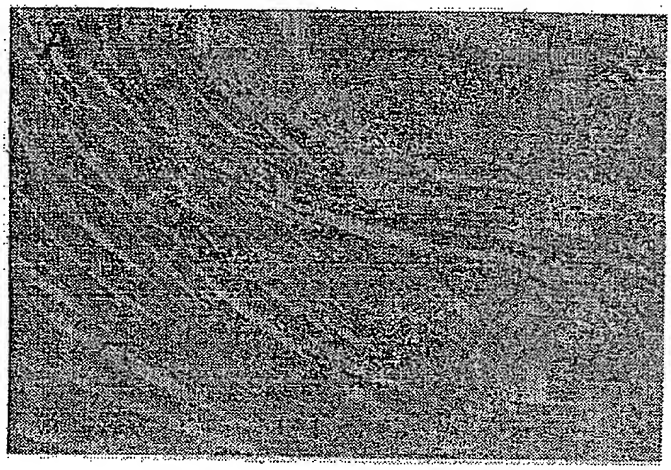


FIG.21A

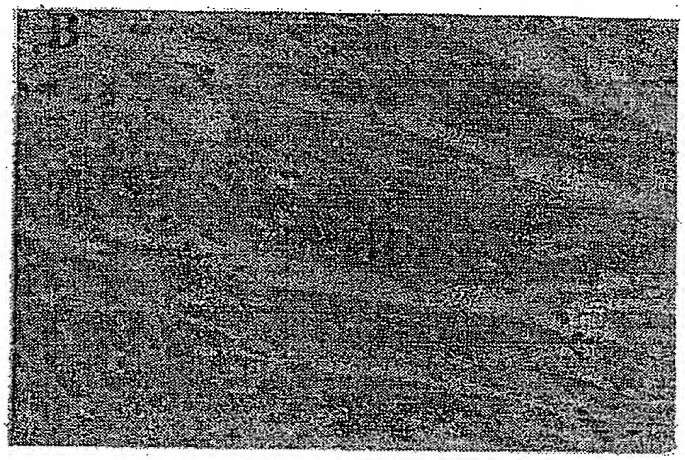


FIG.21B

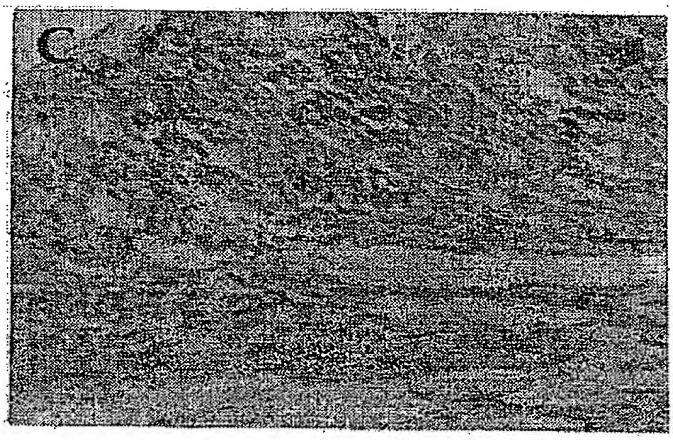


FIG.21C

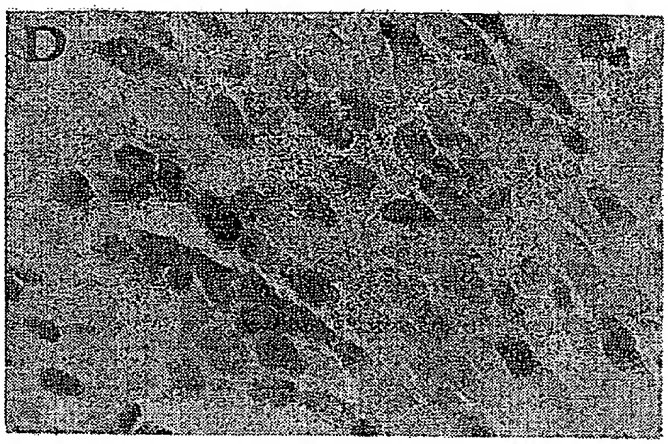


FIG. 21D

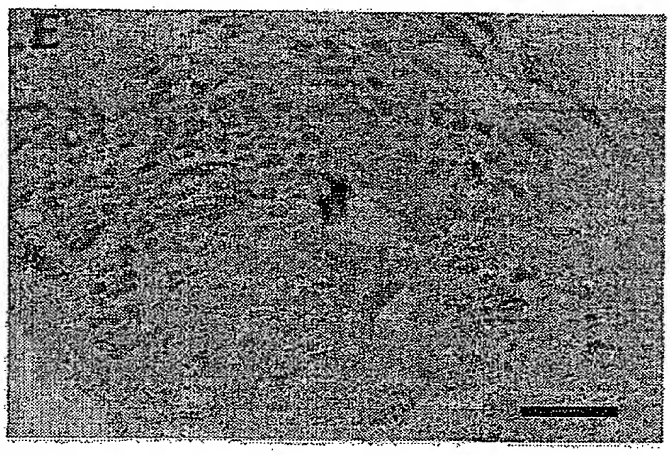


FIG. 21E

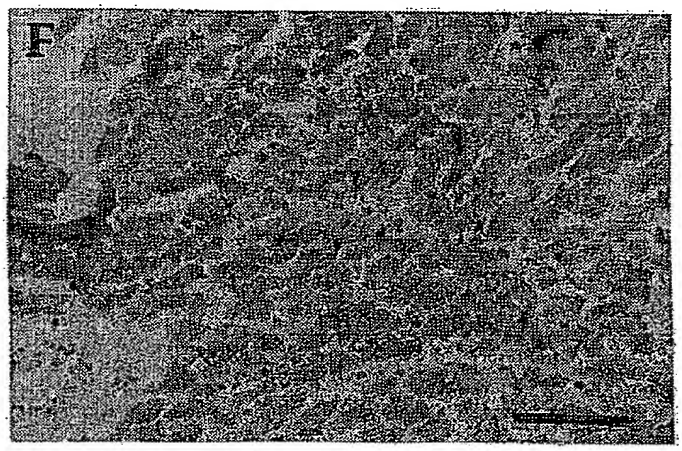


FIG. 21F

Fig. 22

TTCCATCATC AATAATATAC CTTATTTTGG ATTGAAGCCA ATATGATAAT
GAGGGGGTGG 60

AGTTTGTGAC GTGGCGCGGG GCGTGGGAAC GGGGCGGGTG ACGTAGTAGT
GTGGCGGAAG 120

TGTGATGTTG CAAGTGTGGC GGAACACATG TAAGCGACGG ATGTGGCAAA
AGTGACGTTT 180

TTGGTGTGCG CCGGTGTACA CAGGAAGTGA CAATTTTCGC GCGGTTTTAG
GCGGATGTTG 240

TAGTAAATTT GGGCGTAACC GAGTAAGATT TGGCCATTTT CGCGGGAAAA
CTGAATAAGA 300

GGAAGTGAAA TCTGAATAAT TTTGTGTTAC TCATAGCGCG TAATATTTGT
CTAGGGCCTT 360

GCGGCCGCAA GTTGACATTG ATTATTGACT AGTTATTAAT AGTAATCAAT
TACGGGGTCA 420

TTAGTTCATA GCCCATATAT GGAGTTCCGA GTTACATAAC TTACGGTAAA
TGGCCCGCCT 480

GGCTGACCGC CCAACGACCC CCGCCCATTG ACGTCAATAA TGACGTATGT
TCCCATAGTA 540

ACGCGAATAG GGACTTTCCA TTGACGTCAA TGGGTGGAGT ATTTACGGTA
AACTGCCCAC 600

TTGGCAGTAC ATCAAGTGTA TCATATGCCA AGTACGCCCC CTATTGACGT
CAATGACGGT 660

AAATGGCCCG CCTGGCATTA TGCCCAGTAC ATGACCTTAT GGGACTTTCC
TACTTGGCAG 720

TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC GGTTTTGGCA
GTACATCAAT 780

GGGCGTGGAT AGCGGTTTGA CTCACGGGGA TTTCCAAGTC TCCACCCCAT
TGACGTCAAT 840

GGGAGTTTGT TTTGGCACCA AAATCAACGG GACTTTCCAA AATGTCGTAA
CAACTCCGCC 900

CCATTGACGC AAATGGGCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG
CAGAGCTCGC 960

CCGGGGATCC TCTAGAATTC GCTGTCTGCG AGGGCCAGCT GTTGGGGTGA
GTACTCCCTC 1020

TCAAAAGCGG GCATGACTTC TGCGCTAAGA TTGTCAGTTT CCAAAAACGA
GGAGGATTTG 1080

Fig. 22 (continued)

ATATTCACCT GGCCCGCGGT GATGCCTTTG AGGGTGGCCG CGTCCATCTG
GTCAGAAAAG 1140

ACAATCTTTT TGTTGTCAAA AGCGCTTGAG GTGTGGCAGG CTTGAGATCT
GGCCATACAC 1200

TTGAGTGACA ATGACATCCA CTTTGCCTTT CTCTCCACAG GTGTCCACTC
CCAGGTCCAA 1260

CTGCAGCCCC CAAGCTTGGG AATTCTCTCG GAAACGATGA AATATACAAG
TTATATCTTG 1320

GCTTTTCAGC TCTGCATCGT TTTGGGTTCT CTTGGCTGTT ACTGCCAGGA
CCCATATGTA 1380

AAAGAAGCAG AAAACCTTAA GAAATATTTT AATGCAGGTC ATTCAGATGT
AGCGGATAAT 1440

GGAACTCTTT TCTTAGGCAT TTTGAAGAAT TGGAAAGAGG AGAGTGACAG
AAAAATAATG 1500

CAGAGCCAAA TTGTCTCCTT TTACTTCAAA CTTTTTAAAA ACTTTAAAGA
TGACCAGAGC 1560

ATCCAAAAGA GTGTGGAGAC CATCAAGGAA GACATGAATG TCAAGTTTTT
CAATAGCAAC 1620

AAAAAGAAAC GAGATGACTT CGAAAAGCTG ACTAATTATT CGGTAACTGA
CTTGAATGTC 1680

CAACGCAAAG CAATACATGA ACTCATCCAA GTGATGGCTG AACTGTCGCC
AGCAGCTAAA 1740

ACAGGGAAGC GAAAAAGGAG TCAGATGCTG TTTCAAGGTC GAAGAGCATC
CCAGTAATGG 1800

TTGTCCTGCG GATCCCTGCC AGTGGCGCAT AGCGATGCGC GGCAGAACCC
CTTTGATTTT 1860

TAAACGGCGC AGACGGCAAG GGTGGGGGGT AAATAATCAC CCGAGAGTGT
ACAAATAAAA 1920

ACATTTGCCT TTATTGAAAG TGTCTCCTAG TACATTATTT TTACATGTTT
TTCAAGTGAC 1980

AAAAAGAAGT GGCGCTCCTA ATCTGCGCAC TGTGGCTGCG GGAGCTCTAG
AGTCGACGGT 2040

ATCGCCCGAC ATCACCTGTG TCTATGGCCA CTGCCTTGGC TCACAAGTAC
CACTAAACCC 2100

CCTTTCCTGC TCTTGCCTGT GAACAATGGT TAATTGTTCC CAAGAGAGCA
TCTGTCAGTT 2160

Fig. 22 (continued)

GTTGGCAAAA TGATAGACAT TTGAAAATCT GTCTTCTGAC AAATAAAAAG
CATTTATGTT 2220

CACTGCAATG ATGTTTTAAA TTATTTGTCT GTGTCATAGA AGGGTTTATG
CTAAGTTTTC 2280

AAGATACAAA GAAGTGAGGC TTCAGGTCTG ACCTTGGGGA AATAAATGAA
TTACACTTCA 2340

AATTGTGTTG TCAGCTAAGC AGCAGTAGCC ACAGTCTAGC TGAGGGTAAC
TCCAGGGTGC 2400

GCCACAATGT GGCCTCCGAC TGTGGTTGCT TCATGCTAGT GAAAAGCGTG
GCTGTGATTA 2460

AGCATAACAT GGTATGTGGC AACTGCGAGG ACAGGGCCTC TCAGATGCTG
ACCTGCTCGG 2520

ACGGCAACTG TCACCTGCTG AAGACCATTC ACGTAGCCAG CCACTCTCGC
AAGGCCTGGC 2580

CAGTGTTTGA GCATAACATA CTGACCCGCT GTTCCTTGCA TTTGGGTAAC
AGGAGGGGGG 2640

TGTTCCTACC TTACCAATGC AATTTGAGTC ACACTAAGAT ATTGCTTGAG
CCCGAGAGCA 2700

TGTCCAAGGT GAACCTGAAC GGGGTGTTTG ACATGACCAT GAAGATCTGG
AAGGTGCTGA 2760

GGTACGATGA GACCCGCACC AGGTGCAGAC CCTGCGAGTG TGGCGGTAAA
CATATTAGGA 2820

ACCAGCCTGT GATGCTGGAT GTGACCGAGG AGCTGAGGCC CGATCACTTG
GTGCTGGCCT 2880

GCACCCGCGC TGAGTTTGGC TCTAGCGATG AAGATACAGA TTGAGGTACT
GAAATGTGTG 2940

GGCGTGGCTT AAGGGTGGGA AAGAATATAT AAGGTGGGGG TCTTATGTAG
TTTTGTATCT 3000

GTTTTGCAGC AGCCGCCGCC GCCATGAGCA CCAACTCGTT TGATGGAAGC
ATTGTGAGCT 3060

CATATTTGAC AACGCGCATG CCCCCATGGG CCGGGGTGCG TCAGAATGTG
ATGGGCTCCA 3120

GCATTGATGG TCGCCCCGTC CTGCCCGCAA ACTCTACTAC CTTGACCTAC
GAGACCGTGT 3180

CTGGAACGCC GTTGGAGACT GCAGCCTCCG CCGCCGCTTC AGCCGCTGCA
GCCACCGCCC 3240

Fig. 22 (continued)

GCGGGATTGT GACTGACTTT GCTTTCCTGA GCCCGCTTGC AAGCAGTGCA
GCTTCCCGTT 3300

CATCCGCCCG CGATGACAAG TTGACGGCTC TTTTGGCACA ATTGGATTCT
TTGACCCGGG 3360

AACTTAATGT CGTTTCTCAG CAGCTGTTGG ATCTGCGCCA GCAGGTTTCT
GCCCTGAAGG 3420

CTTCCTCCCC TCCCAATGCG GTTTAAAACA TAAATAAAAA ACCAGACTCT
GTTTGGATTT 3480

GGATCAAGCA AGTGTCTTGC TGTCTTTATT TAGGGGTTTT GCGCGCGCGG
TAGGCCCGGG 3540

ACCAGCGGTC TCGGTCGTTG AGGGTCCTGT GTATTTTTTC CAGGACGTGG
TAAAGGTGAC 3600

TCTGGATGTT CAGATACATG GGCATAAGCC CGTCTCTGGG GTGGAGGTAG
CACCACTGCA 3660

GAGCTTCATG CTGCGGGGTG GTGTTGTAGA TGATCCAGTC GTAGCAGGAG
CGCTGGGCGT 3720

GGTGCCTAAA AATGTCTTTC AGTAGCAAGC TGATTGCCAG GGGCAGGCCC
TTGGTGTAAG 3780

TGTTTACAAA GCGGTTAAGC TGGGATGGGT GCATACGTGG GGATATGAGA
TGCATCTTGG 3840

ACTGTATTTT TAGGTTGGCT ATGTTCCCAG CCATATCCCT CCGGGGATTC
ATGTTGTGCA 3900

GAACCACCAG CACAGTGTAT CCGGTGCACT TGGGAAATTT GTCATGTAGC
TTAGAAGGAA 3960

ATGCGTGGAA GAACTTGGAG ACGCCCTTGT GACCTCCAAG ATTTTCCATG
CATTCGTCCA 4020

TAATGATGGC AATGGGCCCA CGGGCGGCGG CCTGGGCGAA GATATTTCTG
GGATCACTAA 4080

CGTCATAGTT GTGTTCCAGG ATGAGATCGT CATAGGCCAT TTTTACAAAG
CGCGGGCGGA 4140

GGGTGCCAGA CTGCGGTATA ATGGTTCCAT CCGGCCCAGG GGCGTAGTTA
CCCTCACAGA 4200

TTTGCATTTC CCACGCTTTG AGTTCAGATG GGGGGATCAT GTCTACCTGC
GGGGCGATGA 4260

AGAAAACGGT TTCCGGGGTA GGGGAGATCA GCTGGGAAGA AAGCAGGTTC
CTGAGCAGCT 4320

Fig. 22 (continued)

GCGACTTACC GCAGCCGGTG GGCCCGTAAA TCACACCTAT TACCGGGTGC
AACTGGTAGT 4380

TAAGAGAGCT GCAGCTGCCG TCATCCCTGA GCAGGGGGGC CACTTCGTTA
AGCATGTCCC 4440

TGACTCGCAT GTTTTCCCTG ACCAAATCCG CCAGAAGGCG CTCGCCGCCC
AGCGATAGCA 4500

GTTCTTGCAA GGAAGCAAAG TTTTTCAACG GTTTGAGACC GTCCGCCGTA
GGCATGCTTT 4560

TGAGCGTTTG ACCAAGCAGT TCCAGGCGGT CCCACAGCTC GGTCACCTGC
TCTACGGCAT 4620

CTCGATCCAG CATATCTCCT CGTTTCGCGG GTTGGGGCGG CTTTCGCTGT
ACGGCAGTAG 4680

TCGGTGCTCG TCCAGACGGG CCAGGGTCAT GTCTTTCCAC GGGCGCAGGG
TCCTCGTCAG 4740

CGTAGTCTGG GTCACGGTGA AGGGGTGCGC TCCGGGCTGC GCGCTGGCCA
GGGTGCGCTT 4800

GAGGCTGGTC CTGCTGGTGC TGAAGCGCTG CCGGTCTTCG CCCTGCGCGT
CGGCCAGGTA 4860

GCATTTGACC ATGGTGTCAT AGTCCAGCCC CTCCGCGGCG TGGCCCTTGG
CGCGCAGCTT 4920

GCCCTTGGAG GAGGCGCCGC ACGAGGGGCA GTGCAGACTT TTGAGGGCGT
AGAGCTTGGG 4980

CGCGAGAAAT ACCGATTCCG GGGAGTAGGC ATCCGCGCCG CAGGCCCCGC
AGACGGTCTC 5040

GCATTCCACG AGCCAGGTGA GCTCTGGCCG TTCGGGGTCA AAAACCAGGT
TTCCCCCATG 5100

CTTTTTGATG CGTTTCTTAC CTCTGGTTTC CATGAGCCGG TGTCCACGCT
CGGTGACGAA 5160

AAGGCTGTCC GTGTCCCCGT ATACAGACTT GAGAGGTCGA GCGATGCCCT
TGAGAGCCTT 5220

CAACCCAGTC AGCTCCTTCC GGTGGGCGCG GGGCATGACT ATCGTCGCCG
CACTTATGAC 5280

TGTCTTCTTT ATCATGCAAC TCGTAGGACA GGTGCCGGCA GCGCTCTGGG
TCATTTTCGG 5340

CGAGGACCGC TTTCGCTGGA GCGCGACGAT GATCGGCCTG TCGCTTGCGG
TATTCGGAAT 5400

Fig. 22 (continued)

CTTGCACGCC CTCGCTCAAG CCTTCGTCAC TGGTCCCGCC ACCAAACGTT
TCGGCGAGAA 5460

GCAGGCCATT ATCGCCGGCA TGGCGGCCGA CGCGCTGGGC TACGTCTTGC
TGGCGTTCGC 5520

GACGCGAGGC TGGATGGCCT TCCCCATTAT GATTCTTCTC GCTTCCGGCG
GCATCGGGAT 5580

GCCCGCGTTG CAGGCCATGC TGTCCAGGCA GGTAGATGAC GACCATCAGG
GACAGCTTCA 5640

AGGATCGCTC GCGGGTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC
TCCGCCCCCC 5700

TGACGAGCAT CACAAAAATC GACGCTCAAG TCAGAGGTGG CGAAACCCGA
CAGGACTATA 5760

AAGATACCAG GCGTTTCCCC CTGGAAGCTC CCTCGTGCGC TCTCCTGTTC
CGACCCTGCC 5820

GCTTACCGGA TACCTGTCCG CCTTTCTCCC TTCGGGAAGC GTGGCGCTTT
CTCAATGCTC 5880

ACGCTGTAGG TATCTCAGTT CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT
GTGTGCACGA 5940

ACCCCCCGTT CAGCCCGACC GCTGCGCCTT ATCCGGTAAC TATCGTCTTG
AGTCCAACCC 6000

GGTAAGACAC GACTTATCGC CACTGGCAGC AGCCACTGGT AACAGGATTA
GCAGAGCGAG 6060

GTATGTAGGC GGTGCTACAG AGTTCTTGAA GTGGTGGCCT AACTACGGCT
ACACTAGAAG 6120

GACAGTATTT GGTATCTGCG CTCTGCTGAA GCCAGTTACC TTCGGAAAAA
GAGTTGGTAG 6180

CTCTTGATCC GGCAAACAAA CCACCGCTGG TAGCGGTGGT TTTTTTGTTT
GCAAGCAGCA 6240

GATTACGCGC AGAAAAAAAG GATCTCAAGA AGATCCTTTG ATCTTTTCTA
CGGGGTCTGA 6300

CGCTCAGTGG AACGAAAACT CACGTTAAGG GATTTTGGTC ATGAGATTAT
CAAAAAGGAT 6360

CTTCACCTAG ATCCTTTTAA ATTAAAAATG AAGTTTTAAA TCAATCTAAA
GTATATATGA 6420

GTAAACTTGG TCTGACAGTT ACCAATGCTT AATCAGTGAG GCACCTATCT
CAGCGATCTG 6480

Fig. 22 (continued)

TCTATTTCGT TCATCCATAG TTGCCTGACT CCCCGTCGTG TAGATAACTA
CGATACGGGA 6540

GGGCTTACCA TCTGGCCCCA GTGCTGCAAT GATACCGCGA GACCCACGCT
CACCGGCTCC 6600

AGATTTATCA GCAATAAACC AGCCAGCCGG AAGGGCCGAG CGCAGAAGTG
GTCCTGCAAC 6660

TTTATCCGCC TCCATCCAGT CTATTAATTG TTGCCGGGAA GCTAGAGTAA
GTAGTTCGCC 6720

AGTTAATAGT TTGCGCAACG TTGTTGCCAT TGCTGCAGGC ATCGTGGTGT
CACGCTCGTC 6780

GTTTGGTATG GCTTCATTCA GCTCCGGTTC CCAACGATCA AGGCGAGTTA
CATGATCCCC 6840

CATGTTGTGC AAAAAAGCGG TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA
GAAGTAAGTT 6900

GGCCGCAGTG TTATCACTCA TGGTTATGGC AGCACTGCAT AATTCTCTTA
CTGTCATGCC 6960

ATCCGTAAGA TGCTTTTCTG TGACTGGTGA GTACTCAACC AAGTCATTCT
GAGAATAGTG 7020

TATGCGGCGA CCGAGTTGCT CTTGCCCGGC GTCAACACGG GATAATACCG
CGCCACATAG 7080

CAGAACTTTA AAAGTGCTCA TCATTGGAAA ACGTTCTTCG GGGCGAAAAC
TCTCAAGGAT 7140

CTTACCGCTG TTGAGATCCA GTTCGATGTA ACCCACTCGT GCACCCAACT
GATCTTCAGC 7200

ATCTTTTACT TTCACCAGCG TTTCTGGGTG AGCAAAAACA GGAAGGCAAA
ATGCCGCAAA 7260

AAAGGGAATA AGGGCGACAC GGAAATGTTG AATACTCATA CTCTTCCTTT
TTCAATATTA 7320

TTGAAGCATT TATCAGGGTT ATTGTCTCAT GAGCGGATAC ATATTTGAAT
GTATTTAGAA 7380

AAATAAACAA ATAGGGGTTC CGCGCACATT TCCCCGAAAA GTGCCACCTG
ACGTCTAAGA 7440

AACCATTATT ATCATGACAT TAACCTATAA AAATAGGCGT ATCACGAGGC
CCTTTCGTCT 7500

TCAAGAA
7507

Fig. 23

```
TTCCATCATC AATAATATAC CTTATTTTGG ATTGAAGCCA ATATGATAAT
GAGGGGGTGG       60

AGTTTGTGAC GTGGCGCGGG GCGTGGGAAC GGGGCGGGTG ACGTAGTAGT
GTGGCGGAAG      120

TGTGATGTTG CAAGTGTGGC GGAACACATG TAAGCGACGG ATGTGGCAAA
AGTGACGTTT      180

TTGGTGTGCG CCGGTGTACA CAGGAAGTGA CAATTTTCGC GCGGTTTTAG
GCGGATGTTG      240

TAGTAAATTT GGGCGTAACC GAGTAAGATT TGGCCATTTT CGCGGGAAAA
CTGAATAAGA      300

GGAAGTGAAA TCTGAATAAT TTTGTGTTAC TCATAGCGCG TAATATTTGT
CTAGGGCCTT      360

GCGGCCGCAA GTTGACATTG ATTATTGACT AGTTATTAAT AGTAATCAAT
TACGGGGTCA      420

TTAGTTCATA GCCCATATAT GGAGTTCCGA GTTACATAAC TTACGGTAAA
TGGCCCGCCT      480

GGCTGACCGC CCAACGACCC CCGCCCATTG ACGTCAATAA TGACGTATGT
TCCCATAGTA      540

ACGCGAATAG GGACTTTCCA TTGACGTCAA TGGGTGGAGT ATTTACGGTA
AACTGCCCAC      600

TTGGCAGTAC ATCAAGTGTA TCATATGCCA AGTACGCCCC CTATTGACGT
CAATGACGGT      660

AAATGGCCCG CCTGGCATTA TGCCCAGTAC ATGACCTTAT GGGACTTTCC
TACTTGGCAG      720

TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC GGTTTTGGCA
GTACATCAAT      780

GGGCGTGGAT AGCGGTTTGA CTCACGGGGA TTTCCAAGTC TCCACCCCAT
TGACGTCAAT      840

GGGAGTTTGT TTTGGCACCA AAATCAACGG GACTTTCCAA AATGTCGTAA
CAACTCCGCC      900

CCATTGACGC AAAGGGTCGG TAGGCGTGTA CGGTGGGAGG TCTATATAAG
CAGAGCTCGC      960

CCGGGGATCC TCTAGAATTC GCTGTCTGCG AGGGCCAGCT GTTGGGGTGA
GTACTCCCTC     1020
```

Fig. 23 (continued)

TCAAAAGCGG GCATGACTTC TGCGCTAAGA TTGTCAGTTT CCAAAAACGA
GGAGGATTTG 1080

ATATTCACCT GGCCCGCGGT GATGCCTTTG AGGGTGGCCG CGTCCATCTG
GTCAGAAAAG 1140

ACAATCTTTT TGTTGTCAAA AGCGCTTGAG GTGTGGCAGG CTTGAGATCT
GGCCATACAC 1200

TTGAGTGACA ATGACATCCA CTTTGCCTTT CTCTCCACAG GTGTCCACTC
CCAGGTCCAA 1260

CTGCAGCCCC CAAGCTTGGT ACCGGTGATC AGATATCTCG AGGTACCGTC
GACGGTATCG 1320

CCCGACATCA CCTGTGTCTA TGGCCACTGC CTTGGCTCAC AAGTACCACT
AAACCCCCTT 1380

TCCTGCTCTT GCCTGTGAAC AATGGTTAAT TGTTCCCAAG AGAGCATCTG
TCAGTTGTTG 1440

GCAAAATGAT AGACATTTGA AAATCTGTCT TCTGACAAAT AAAAAGCATT
TATGTTCACT 1500

GCAATGATGT TTTAAATTAT TTGTCTGTGT CATAGAAGGG TTTATGCTAA
GTTTTCAAGA 1560

TACAAAGAAG TGAGGCTTCA GGTCTGACCT TGGGGAAATA AATGAATTAC
ACTTCAAATT 1620

GTGTTGTCAG CTAAGCAGCA GTAGCCACAG TCTAGCTGAG GGTAACTCCA
GGGTGCGCCA 1680

CAATGTGGCC TCCGACTGTG GTTGCTTCAT GCTAGTGAAA AGCGTGGCTG
TGATTAAGCA 1740

TAACATGGTA TGTGGCAACT GCGAGGACAG GGCCTCTCAG ATGCTGACCT
GCTCGGACGG 1800

CAACTGTCAC CTGCTGAAGA CCATTCACGT AGCCAGCCAC TCTCGCAAGG
CCTGGCCAGT 1860

GTTTGAGCAT AACATACTGA CCCGCTGTTC CTTGCATTTG GGTAACAGGA
GGGGGGTGTT 1920

CCTACCTTAC CAATGCAATT TGAGTCACAC TAAGATATTG CTTGAGCCCG
AGAGCATGTC 1980

CAAGGTGAAC CTGAACGGGG TGTTTGACAT GACCATGAAG ATCTGGAAGG
TGCTGAGGTA 2040

Fig. 23 (continued)

CGATGAGACC CGCACCAGGT GCAGACCCTG CGAGTGTGGC GGTAAACATA
TTAGGAACCA 2100

GCCTGTGATG CTGGATGTGA CCGAGGAGCT GAGGCCCGAT CACTTGGTGC
TGGCCTGCAC 2160

CCGCGCTGAG TTTGGCTCTA GCTATGAAGA TACAGATTGA GGTACTGAAA
TGTGTGGGCG 2220

TGGCTTAAGG GTGGGAAAGA ATATATAAGG TGGGGGTCTT ATGTAGTTTT
GTATCTGTTT 2280

TGCAGCAGCC GCCGCCGCCA TGAGCACCAA CTCGTTTGAT GGAAGCATTG
TGAGCTCATA 2340

TTTGACAACG CGCATGCCCC CATGGGCCGG GGTGCGTCAG AATGTGATGG
GCTCCAGCAT 2400

TGATGGTCGC CCCGTCCTGC CCGCAAACTC TACTACCTTG ACCTACGAGA
CCGTGTCTGG 2460

AACGCCGTTG GAGACTGCAG CCTCCGCCGC CGCTTCAGCC GCTGCAGCCA
CCGCCCGCGG 2520

GATTGTGACT GACTTTGCTT TCCTGAGCCC GCTTGCAAGC AGTGCAGCTT
CCCGTTCATC 2580

CGCCCGCGAT GACAAGTTGA CGGCTCTTTT GGCACAATTG GATTCTTTGA
CCCGGGAACT 2640

TAATGTCGTT TCTCAGCAGC TGTTGGATCT GCGCCAGCAG GTTTCTGCCC
TGAAGGCTTC 2700

CTCCCCTCCC AATGCGGTTT AAAACATAAA TAAAAAACCA GACTCTGTTT
GGATTTGGAT 2760

CAAGCAAGTG TCTTGCTGTC TTTATTTAGG GGTTTTGCGC GCGCGGTAGG
CCCGGGACCA 2820

GCGGTCTCGG TCGTTGAGGG TCCTGTGTAT TTTTTCCAGG ACGTGGTAAA
GGTGACTCTG 2880

GATGTTCAGA TACATGGGCA TAAGCCCGTC TCTGGGGTGG AGGTAGCACC
ACTGCAGAGC 2940

TTCATGCTGC GGGGTGGTGT TGTAGATGAT CCAGTCGTAG CAGGAGCGCT
GGGCGTGGTG 3000

CCTAAAAATG TCTTTCAGTA GCAAGCTGAT TGCCAGGGGC AGGCCCTTGG
TGTAAGTGTT 3060

Fig. 23 (continued)

TACAAAGCGG TTAAGCTGGG ATGGGTGCAT ACGTGGGGAT ATGAGATGCA
TCTTGGACTG 3120

TATTTTTAGG TTGGCTATGT TCCCAGCCAT ATCCCTCCGG GGATTCATGT
TGTGCAGAAC 3180

CACCAGCACA GTGTATCCGG TGCACTTGGG AAATTTGTCA TGTAGCTTAG
AAGGAAATGC 3240

GTGGAAGAAC TTGGAGACGC CCTTGTGACC TCCAAGATTT TCCATGCATT
CGTCCATAAT 3300

GATGGCAATG GGCCCACGGG CGGCGGCCTG GGCGAAGATA TTTCTGGGAT
CACTAACGTC 3360

ATAGTTGTGT TCCAGGATGA GATCGTCATA GGCCATTTTT ACAAAGCGCG
GGCGGAGGGT 3420

GCCAGACTGC GGTATAATGG TTCCATCCGG CCCAGGGGCG TAGTTACCCT
CACAGATTTG 3480

CATTTCCCAC GCTTTGAGTT CAGATGGGGG GATCATGTCT ACCTGCGGGG
CGATGAAGAA 3540

AACGGTTTCC GGGGTAGGGG AGATCAGCTG GGAAGAAAGC AGGTTCCTGA
GCAGCTGCGA 3600

CTTACCGCAG CCGGTGGGCC CGTAAATCAC ACCTATTACC GGGTGCAACT
GGTAGTTAAG 3660

AGAGCTGCAG CTGCCGTCAT CCCTGAGCAG GGGGGCCACT TCGTTAAGCA
TGTCCCTGAC 3720

TCGCATGTTT TCCCTGACCA AATCCGCCAG AAGGCGCTCG CCGCCCAGCG
ATAGCAGTTC 3780

TTGCAAGGAA GCAAAGTTTT TCAACGGTTT GAGACCGTCC GCCGTAGGCA
TGCTTTTGAG 3840

CGTTTGACCA AGCAGTTCCA GGCGGTCCCA CAGCTCGGTC ACCTGCTCTA
CGGCATCTCG 3900

ATCCAGCATA TCTCCTCGTT TCGCGGGTTG GGGCGGCTTT CGCTGTACGG
CAGTAGTCGG 3960

TGCTCGTCCA GACGGGCCAG GGTCATGTCT TTCCACGGGC GCAGGGTCCT
CGTCAGCGTA 4020

GTCTGGGTCA CGGTGAAGGG GTGCGCTCCG GGCTGCGCGC TGGCCAGGGT
GCGCTTGAGG 4080

Fig. 23 (continued)

CTGGTCCTGC TGGTGCTGAA GCGCTGCCGG TCTTCGCCCT GCGCGTCGGC
CAGGTAGCAT 4140

TTGACCATGG TGTCATAGTC CAGCCCCTCC GCGGCGTGGC CCTTGGCGCG
CAGCTTGCCC 4200

TTGGAGGAGG CGCCGCACGA GGGGCAGTGC AGACTTTTGA GGGCGTAGAG
CTTGGGCGCG 4260

AGAAATACCG ATTCCGGGGA GTAGGCATCC GCGCCGCAGG CCCCGCAGAC
GGTCTCGCAT 4320

TCCACGAGCC AGGTGAGCTC TGGCCGTTCG GGGTCAAAAA CCAGGTTTCC
CCCATGCTTT 4380

TTGATGCGTT TCTTACCTCT GGTTTCCATG AGCCGGTGTC CACGCTCGGT
GACGAAAAGG 4440

CTGTCCGTGT CCCCGTATAC AGACTTGAGA GGTCGAGCGA TGCCCTTGAG
AGCCTTCAAC 4500

CCAGTCAGCT CCTTCCGGTG GGCGCGGGGC ATGACTATCG TCGCCGCACT
TATGACTGTC 4560

TTCTTTATCA TGCAACTCGT AGGACAGGTG CCGGCAGCGC TCTGGGTCAT
TTTCGGCGAG 4620

GACCGCTTTC GCTGGAGCGC GACGATGATC GGCCTGTCGC TTGCGGTATT
CGGAATCTTG 4680

CACGCCCTCG CTCAAGCCTT CGTCACTGGT CCCGCCACCA AACGTTTCGG
CGAGAAGCAG 4740

GCCATTATCG CCGGCATGGC GGCCGACGCG CTGGGCTACG TCTTGCTGGC
GTTCGCGACG 4800

CGAGGCTGGA TGGCCTTCCC CATTATGATT CTTCTCGCTT CCGGCGGCAT
CGGGATGCCC 4860

GCGTTGCAGG CCATGCTGTC CAGGCAGGTA GATGACGACC ATCAGGGACA
GCTTCAAGGA 4920

TCGCTCGCGG GTAAAAAGGC CGCGTTGCTG GCGTTTTTCC ATAGGCTCCG
CCCCCCTGAC 4980

GAGCATCACA AAAATCGACG CTCAAGTCAG AGGTGGCGAA ACCCGACAGG
ACTATAAAGA 5040

TACCAGGCGT TTCCCCCTGG AAGCTCCCTC GTGCGCTCTC CTGTTCCGAG
CCTGCCGCTT 5100

Fig. 23 (continued)

ACCGGATACC TGTCCGCCTT TCTCCCTTCG GGAAGCGTGG CGCTTTCTCA
ATGCTCACGC 5160

TGTAGGTATC TCAGTTCGGT GTAGGTCGTT CGCTCCAAGC TGGGCTGTGT
GCACGAACCC 5220

CCCGTTCAGC CCGACCGCTG CGCCTTATCC GGTAACTATC GTCTTGAGTC
CAACCCGGTA 5280

AGACACGACT TATCGCCACT GGCAGCAGCC ACTGGTAACA GGATTAGCAG
AGCGAGGTAT 5340

GTAGGCGGTG CTACAGAGTT CTTGAAGTGG TGGCCTAACT ACGGCTACAC
TAGAAGGACA 5400

GTATTTGGTA TCTGCGCTCT GCTGAAGCCA GTTACCTTCG GAAAAAGAGT
TGGTAGCTCT 5460

TGATCCGGCA AACAAACCAC CGCTGGTAGC GGTGGTTTTT TTGTTTGCAA
GCAGCAGATT 5520

ACGCGCAGAA AAAAAGGATC TCAAGAAGAT CCTTTGATCT TTTCTACGGG
GTCTGACGCT 5580

CAGTGGAACG AAAACTCACG TTAAGGGATT TTGGTCATGA GATTATCAAA
AAGGATCTTC 5640

ACCTAGATCC TTTTAAATTA AAAATGAAGT TTTAAATCAA TCTAAAGTAT
ATATGAGTAA 5700

ACTTGGTCTG ACAGTTACCA ATGCTTAATC AGTGAGGCAC CTATCTCAGC
GATCTGTCTA 5760

TTTCGTTCAT CCATAGTTGC CTGACTCCCC GTCGTGTAGA TAACTACGAT
ACGGGAGGGC 5820

TTACCATCTG GCCCCAGTGC TGCAATGATA CCGCGAGACC CACGCTCACC
GGCTCCAGAT 5880

TTATCAGCAA TAAACCAGCC AGCCGGAAGG GCCGAGCGCA GAAGTGGTCC
TGCAACTTTA 5940

TCCGCCTCCA TCCAGTCTAT TAATTGTTGC CGGGAAGCTA GAGTAAGTAG
TTCGCCAGTT 6000

AATAGTTTGC GCAACGTTGT TGCCATTGCT GCAGGCATCG TGGTGTCACG
CTCGTCGTTT 6060

GGTATGGCTT CATTCAGCTC CGGTTCCCAA CGATCAAGGC GAGTTACATG
ATCCCCCATG 6120

Fig. 23 (continued)

TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT CCTCCGATCG TTGTCAGAAG
TAAGTTGGCC 6180

GCAGTGTTAT CACTCATGGT TATGGCAGCA CTGCATAATT CTCTTACTGT
CATGCCATCC 6240

GTAAGATGCT TTTCTGTGAC TGGTGAGTAC TCAACCAAGT CATTCTGAGA
ATAGTGTATG 6300

CGGCGACCGA GTTGCTCTTG CCCGGCGTCA ACACGGGATA ATACCGCGCC
ACATAGCAGA 6360

ACTTTAAAAG TGCTCATCAT TGGAAAACGT TCTTCGGGGC GAAAACTCTC
AAGGATCTTA 6420

CCGCTGTTGA GATCCAGTTC GATGTAACCC ACTCGTGCAC CCAACTGATC
TTCAGCATCT 6480

TTTACTTTCA CCAGCGTTTC TGGGTGAGCA AAAACAGGAA GGCAAAATGC
CGCAAAAAAG 6540

GGAATAAGGG CGACACGGAA ATGTTGAATA CTCATACTCT TCCTTTTTCA
ATATTATTGA 6600

AGCATTTATC AGGGTTATTG TCTCATGAGC GGATACATAT TTGAATGTAT
TTAGAAAAAT 6660

AAACAAATAG GGGTTCCGCG CACATTTCCC CGAAAAGTGC CACCTGACGT
CTAAGAAACC 6720

ATTATTATCA TGACATTAAC CTATTAAAAT AGGCGTATCA CGAGGCCCTT
TCGTCTTCAA 6780

GAA
6783

INTERNATIONAL SEARCH REPORT

In...national application No.
PCT/US94/14502

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/10, 15/63, 15/86; C12P 21/00
US CL :435/172.3, 240.2, 320.1; 800/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 240.2, 320.1; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG DATABASES: BIOSIS PREVIEWS, MEDLINE, WORLD PATENT INDEX, CA SEARCH; APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature genetics, Volume 5, issued December 1993, T. A. G. Smith et al., "Adenovirus mediated expression of therapeutic plasma levels of human factor IX in mice," pages 397-402, see page 401 under Methodology.	1-16
Y	Current Opinion in Genetics and Development, Volume 3, Number 3, issued June 1993, K. F. Kozarsky et al., "Gene therapy: adenovirus vectors," pages 499-503, see entire article.	1-16
Y	J. Clin. Invest., Volume 90, issued August 1992, L. D. Stratford-Perricaudet et al., "Widespread Long-term Gene Transfer to Mouse Skeletal Muscles and Heart," pages 626-630, see entire article.	1-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

19 FEBRUARY 1995

Date of mailing of the international search report

03 MAR 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JOHNNY F. RAILEY II, PH.D.

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/14502

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Clin. Invest., Volume 92, issued July 1993, L. A. Kirshenbaum et al., "Highly Efficient Gene Transfer into Adult Ventricular Myocytes by Recombinant Adenovirus," pages 381-387, see entire article.	1-16
Y	Proc. Natl. Acad. Sci. USA, Volume 89, issued April 1992, B. Quantin et al., "Adenovirus as an expression vector in muscle cells in vivo," pages 2581-2584, see entire article.	1-16
P,X	Proc. Natl. Acad. Sci. USA, Volume 90, issued 15 December 1993, A. Kass-Eisler et al., "Quantitative determination of adenovirus-mediated gene delivery to rat cardiac myocytes in vitro and in vivo," pages 11498-11502, see entire article.	1-16